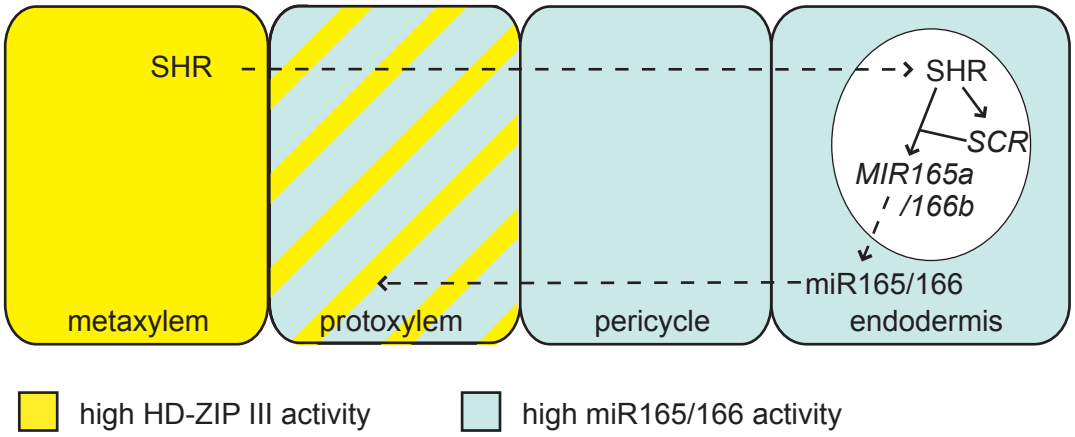
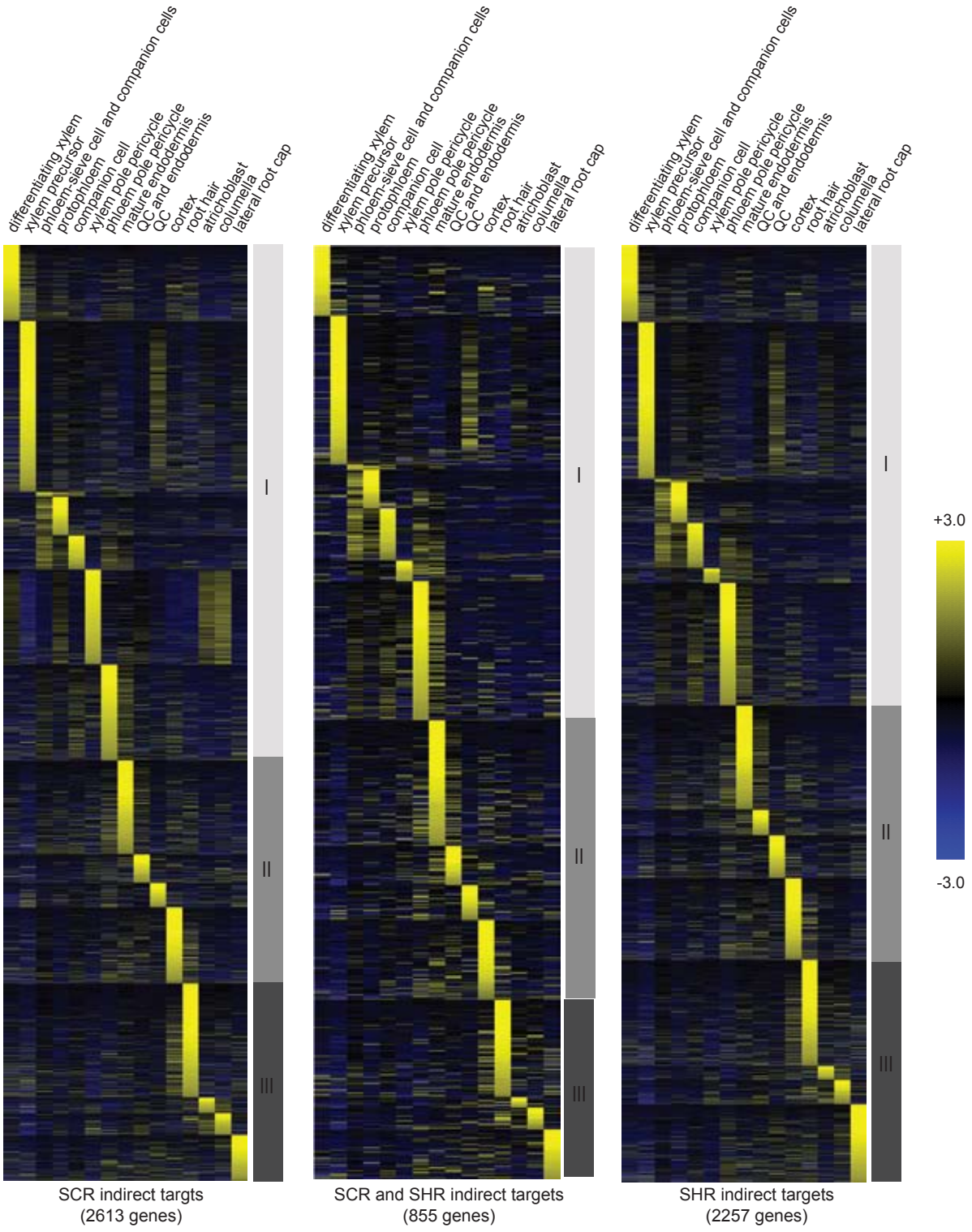
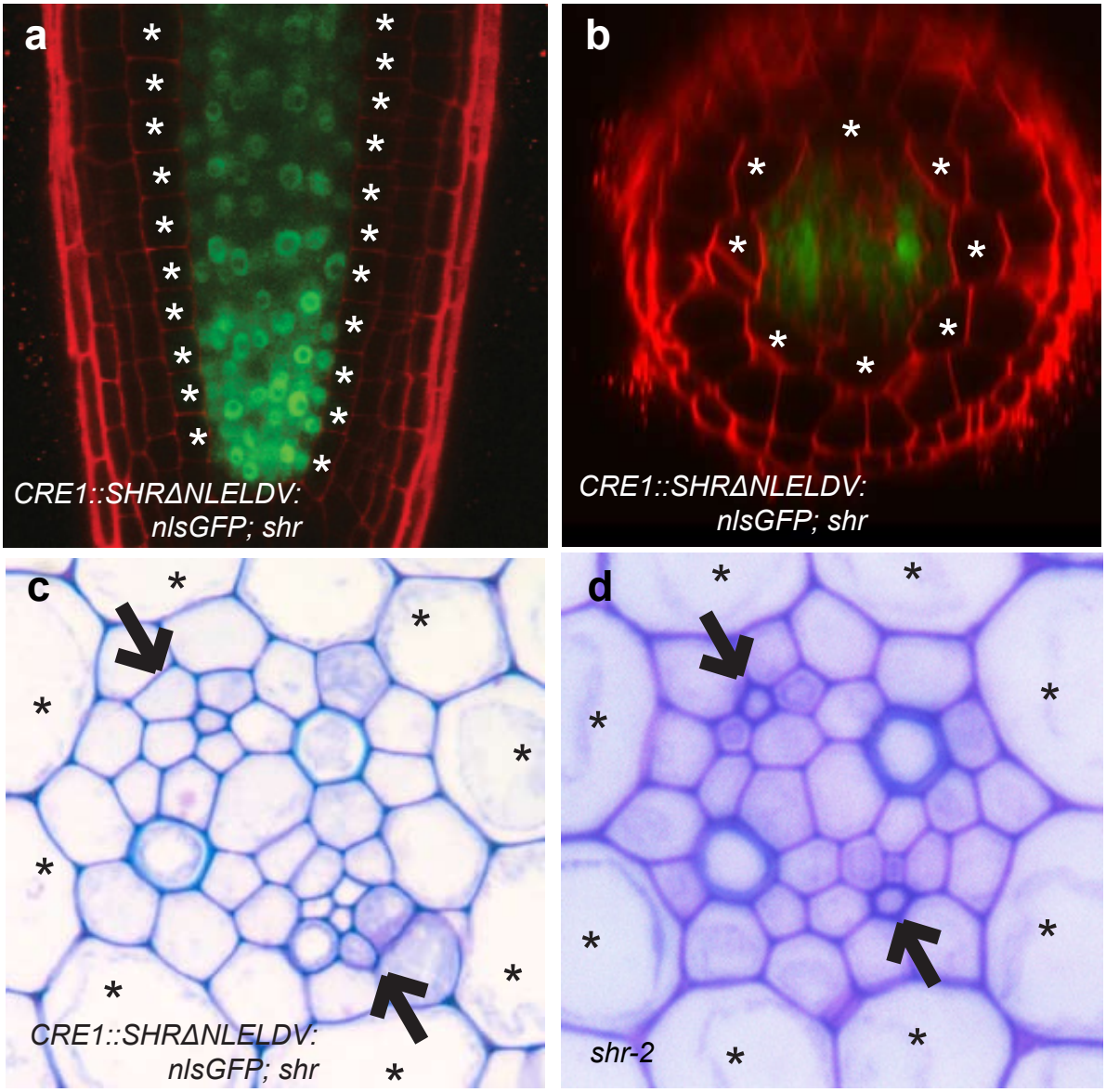
SUPPLEMENTARY INFORMATION




**Supplementary Figure 1. Molecular model of miR165/6 and SHR movements in xylem patterning.**

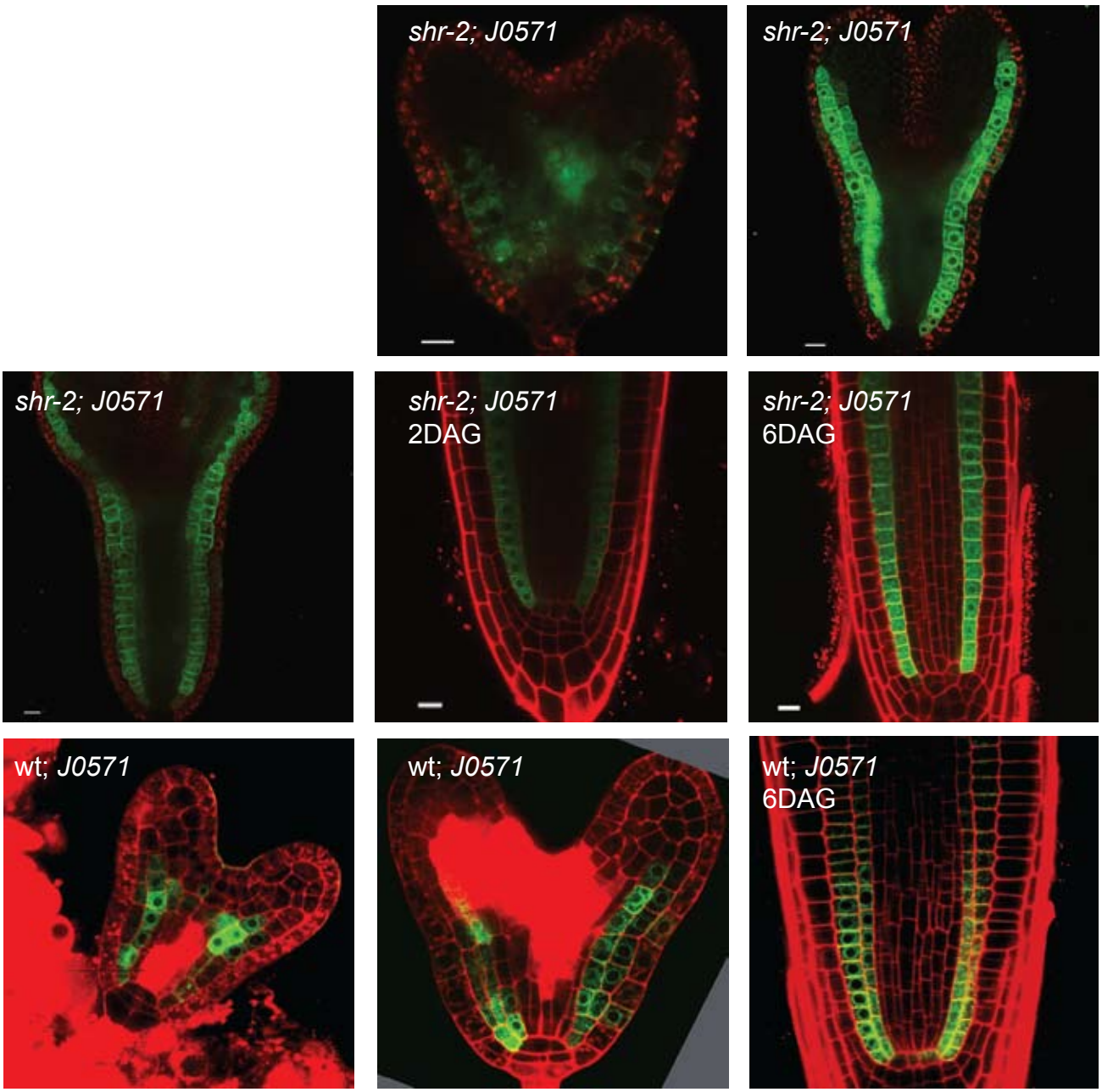



**Supplementary Figure 2. SHR and SCR co-regulate genes highly expressed in the stele cell types.** One third of the genes that are directly or indirectly co-regulated by SHR and SCR (on-line methods) are highly enriched in the endodermis, cortex, and QC (Group II) and a half of them are enriched in the stele, more specifically in xylem precursors and phloem-pole pericycle (Group I). Group I: genes with the highest expression in the stele cell types. Group II: genes with the highest expression in the ground tissues and QC. Group III: genes with the highest expression in the epidermis and root cap.



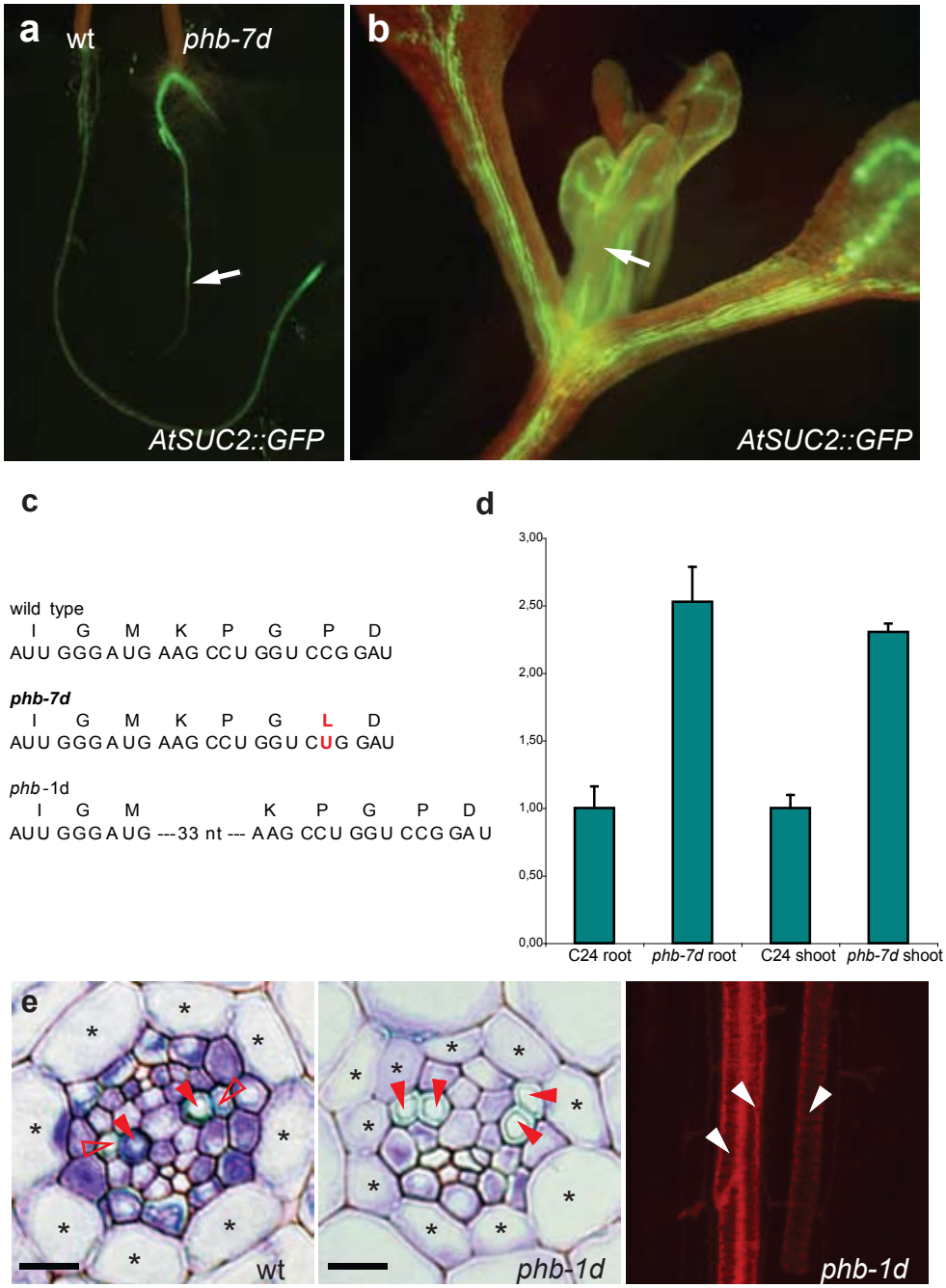

**Supplementary Figure 3. Non-mobile SHR expressed in the root stele drives cell division in the phloem axis.**

**a**, *CRE1::SHRΔNLELDV:nlsGFP* localization in the longitudinal axis of the *shr-2* root. **b,** *CRE1::SHRΔNLELDV:nlsGFP* localization in a cross-section of the root. Movement of *SHRΔNLELDV:nlsGFP* into the ground tissue layer is not observed. **c,** Cross-section of a *CRE1::SHRΔNLELDV:nlsGFP* transgenic root shows more cells in the phloem poles (pointed by arrows) in comparison to *shr-2* (**d**). * indicates the ground tissue layer.



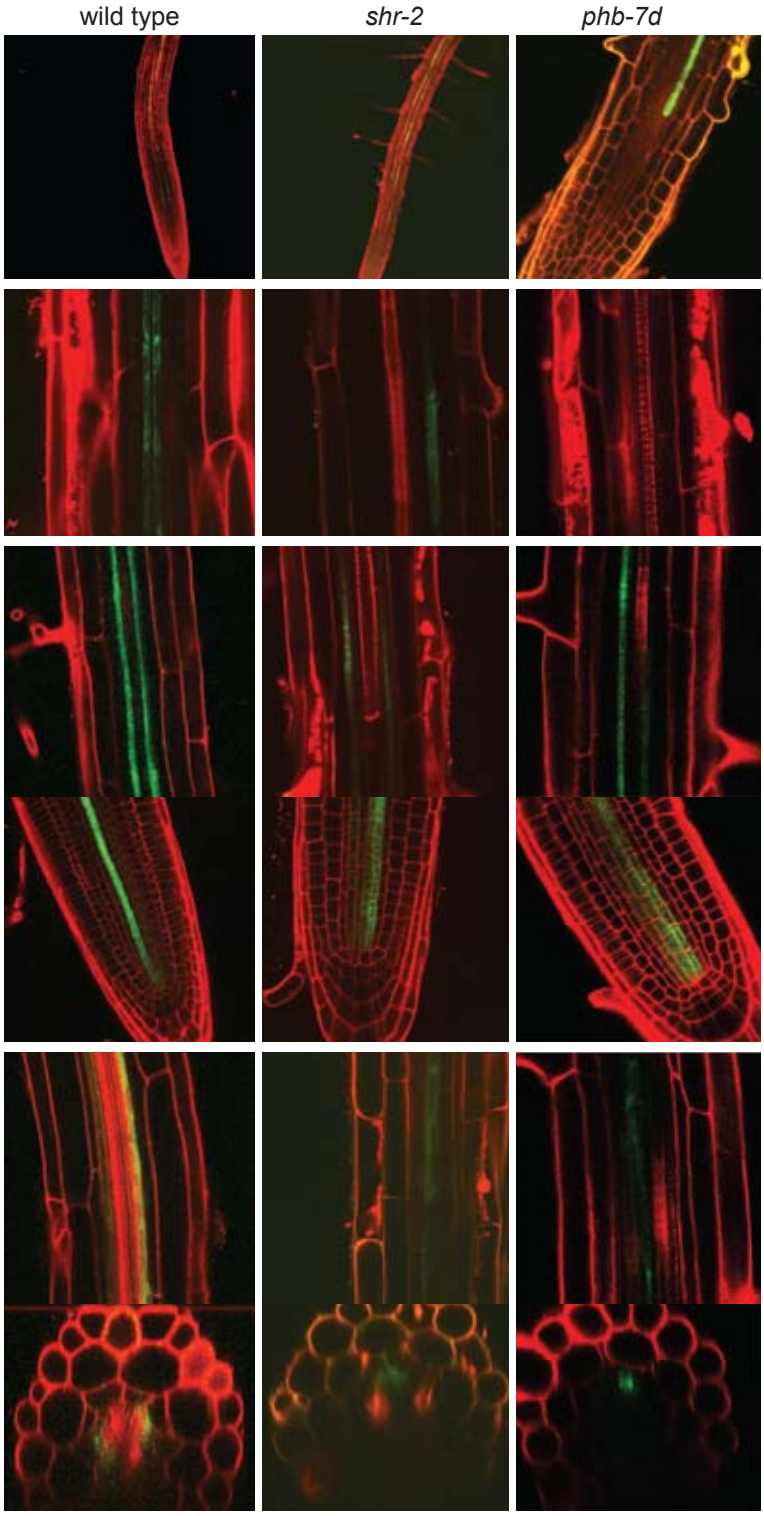

**Supplementary Figure 4. *J0571>>GFP* expression pattern in *shr-2* and wild type roots.**

*J0571>>GFP* expression starts in the heart stage embryo and quickly becomes restricted in the ground tissue layer. During embryogenesis, GFP expression in the QC is not observed in either *shr-2* or wild type roots. However, GFP in the wild type seedling root at 6 days after germination (DAG) shows sporadic expression in the QC. The expression in the QC region is not observed in the *shr-2* root. Scale bar is 10 μm.



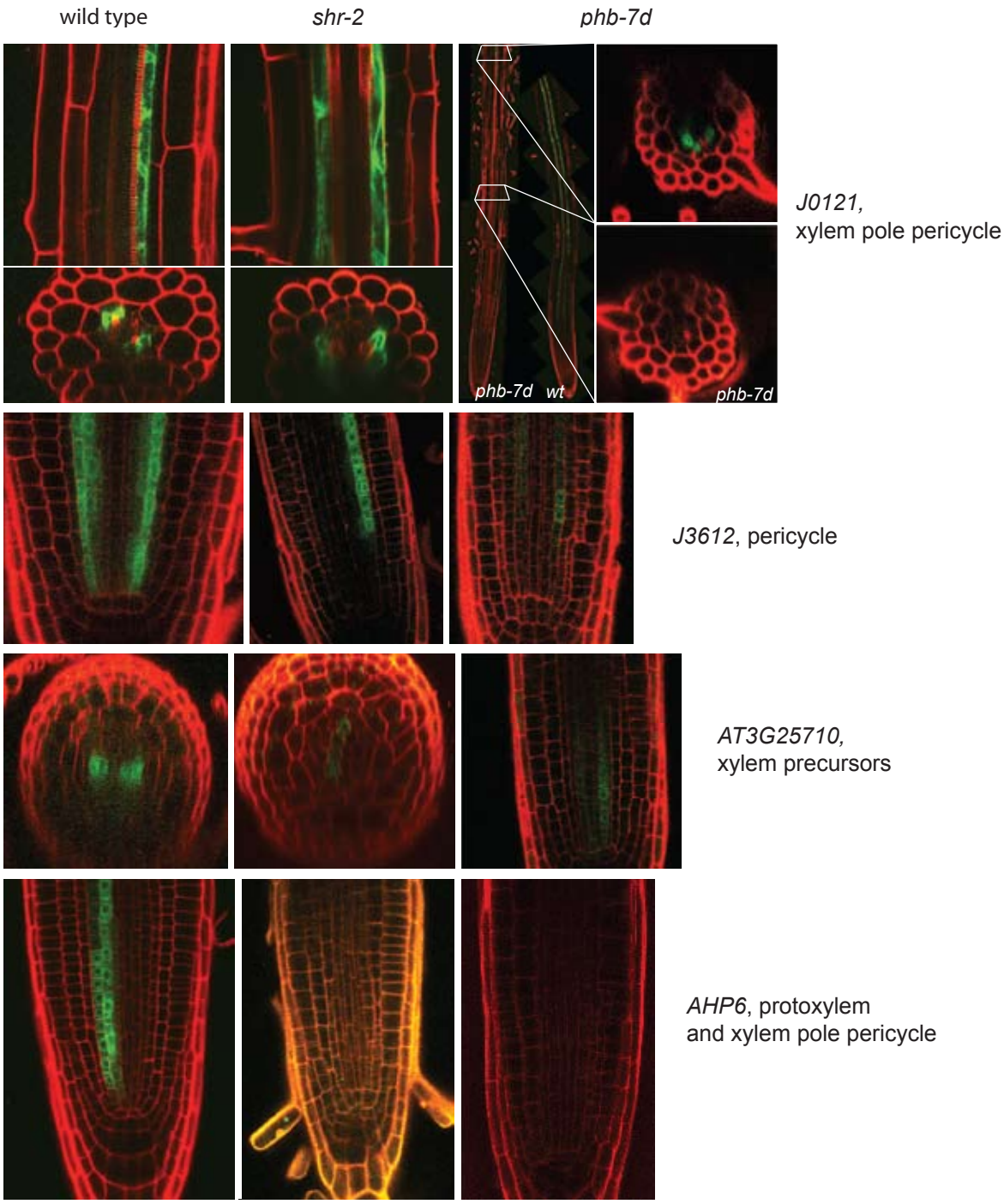

**Supplementary Figure 5. Identification and cloning of *phb-7d*.**

**a**, The EMS generated *phb-7d* mutant was identified as having a delayed expression of the *AtSUC2::GFP* [41] phloem marker and a short root. **b**, *phb-7d* has radialized first two leaves that often have no vascular tissues as detected by absence of *AtSUC2::GFP* expression and histological analyses (results not shown). The leaf phenotype is inherited in a semi-dominant manner. **c**, The *phb-7d* mutant has a C to T base-pair substitution in the miRNA binding site of *PHB* resulting in a P to L amino acid substitution. **d**, Real-time RT-PCR of *PHB* expression in *phb-7d* compared to wt. Error bars show means ± s.d. n= 4 technical repeats. **e**, *phb-1d* has a similar vascular phenotype as *phb-7d* with invariably ectopic metaxylem formation in the protoxylem position. Asterisks mark endodermis, filled arrowheads indicate metaxylem, and unfilled arrowheads indicate protoxylem. Scale bar is 10 μm.

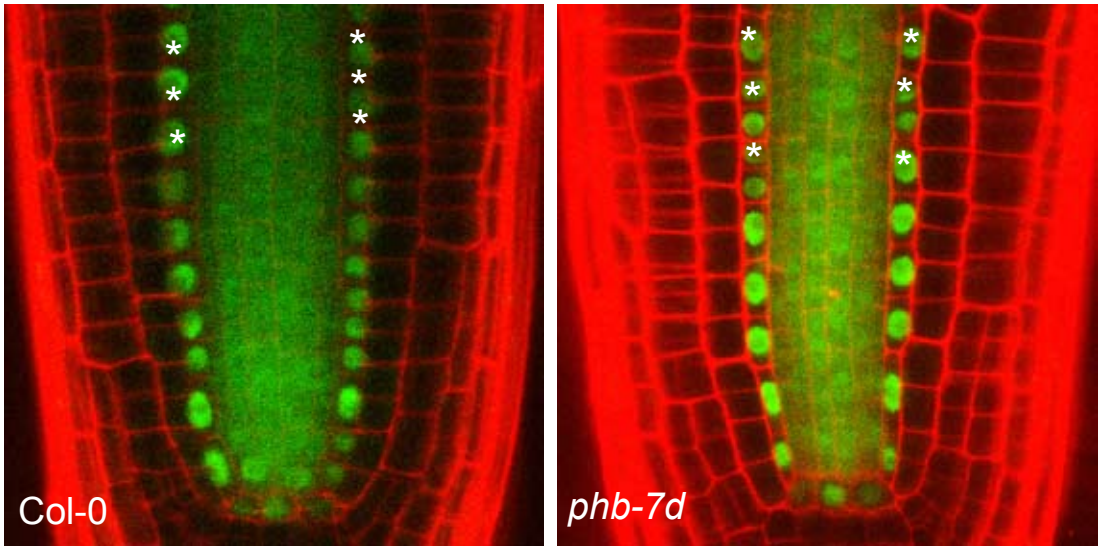



**Supplementary Figure S6_1. Comparison between *shr-2* and *phb-7d* using vascular cell type specific GFP markers listed in Table S1.**

The similarity of *shr-2* and *phb-7d* phenotypes includes most aspects of vascular development. In addition to ectopic metaxylem in protoxylem position there is delayed formation of phloem. There is also a delay in pericycle formation both mutants, but this is more pronounced in *phb-7d* compared to *shr-2*, as seen by marker analyses.




**Supplementary Figure S6_2. Comparison between *shr-2* and *phb-7d* using vascular cell type specific GFP markers listed in Table S1.**



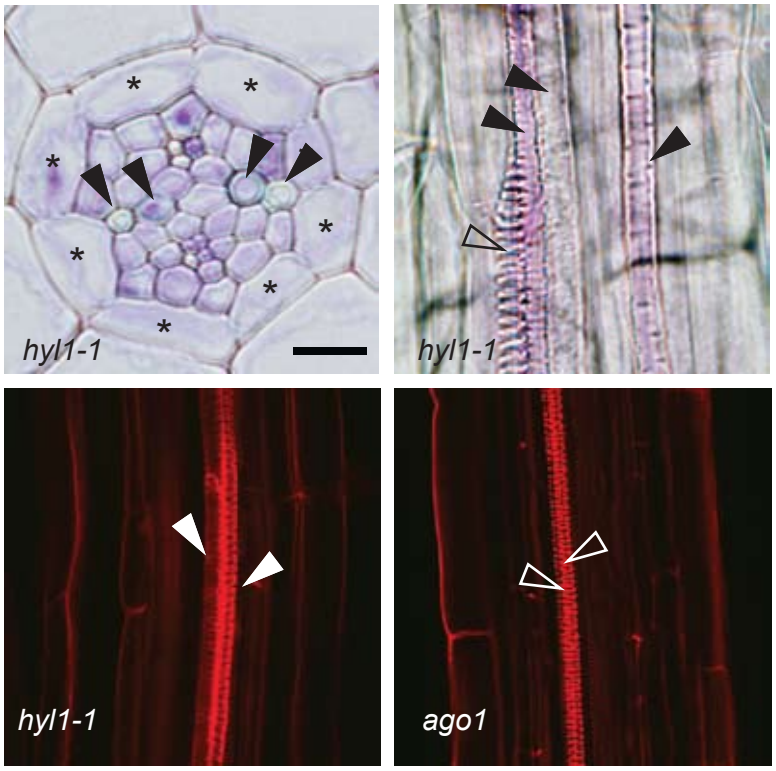

**Supplementary Figure 7. Comparison of SHR protein localization in wt and *phb-7d* using *SHR*::*SHR*:GFP [13].**

The *SHR*::*SHR*:GFP marker displayed a similar pattern in *phb-7d* as in wt. Asterisks mark the endodermis.

**a**

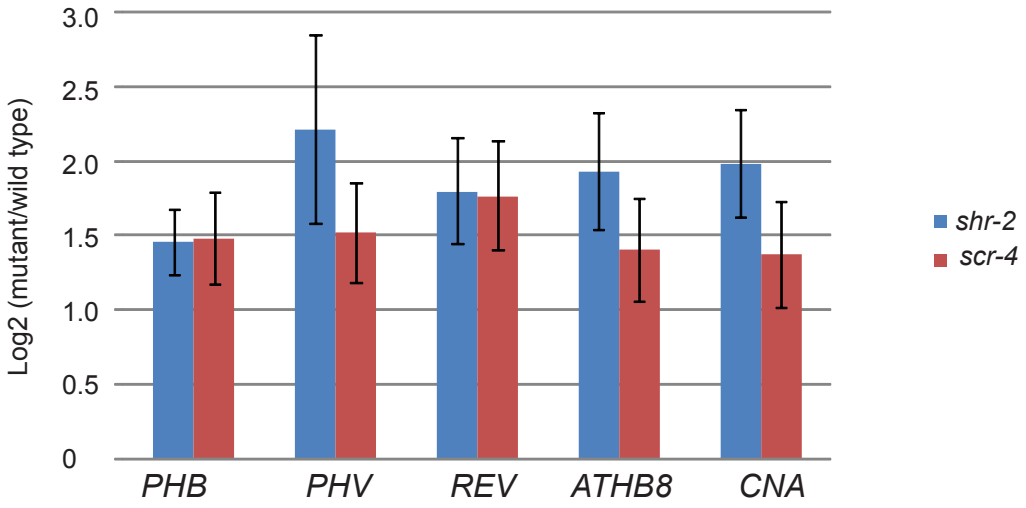
| Genotype | Phenotype |
|---|---|
| *ago1-3* | Occasional double protoxylem |
| *ago1-8* | Wild type protoxylem and metaxylem pattern |
| *zll-3* | Occasional double protoxylem |
| *dcl1-7* | Occasional ectopic xylem in the pericycle |
| *ago7* | Wild type protoxylem and metaxylem pattern |
| *hyl1-1* | Ectopic xylem in pericycle and metaxylem in protoxylem position |
| *sgs3* | Wild type protoxylem and metaxylem pattern |
| *arf3 ett* | Wild type protoxylem and metaxylem pattern |
| *hst-15* | Wild type protoxylem and metaxylem pattern |
| *dcl4* | Occasional double protoxylem |
| *hen1-1* | Wild type protoxylem and metaxylem pattern |
| *hen1-5* | Wild type protoxylem and metaxylem pattern |
| *rdr6* | Wild type protoxylem and metaxylem pattern |

**b**



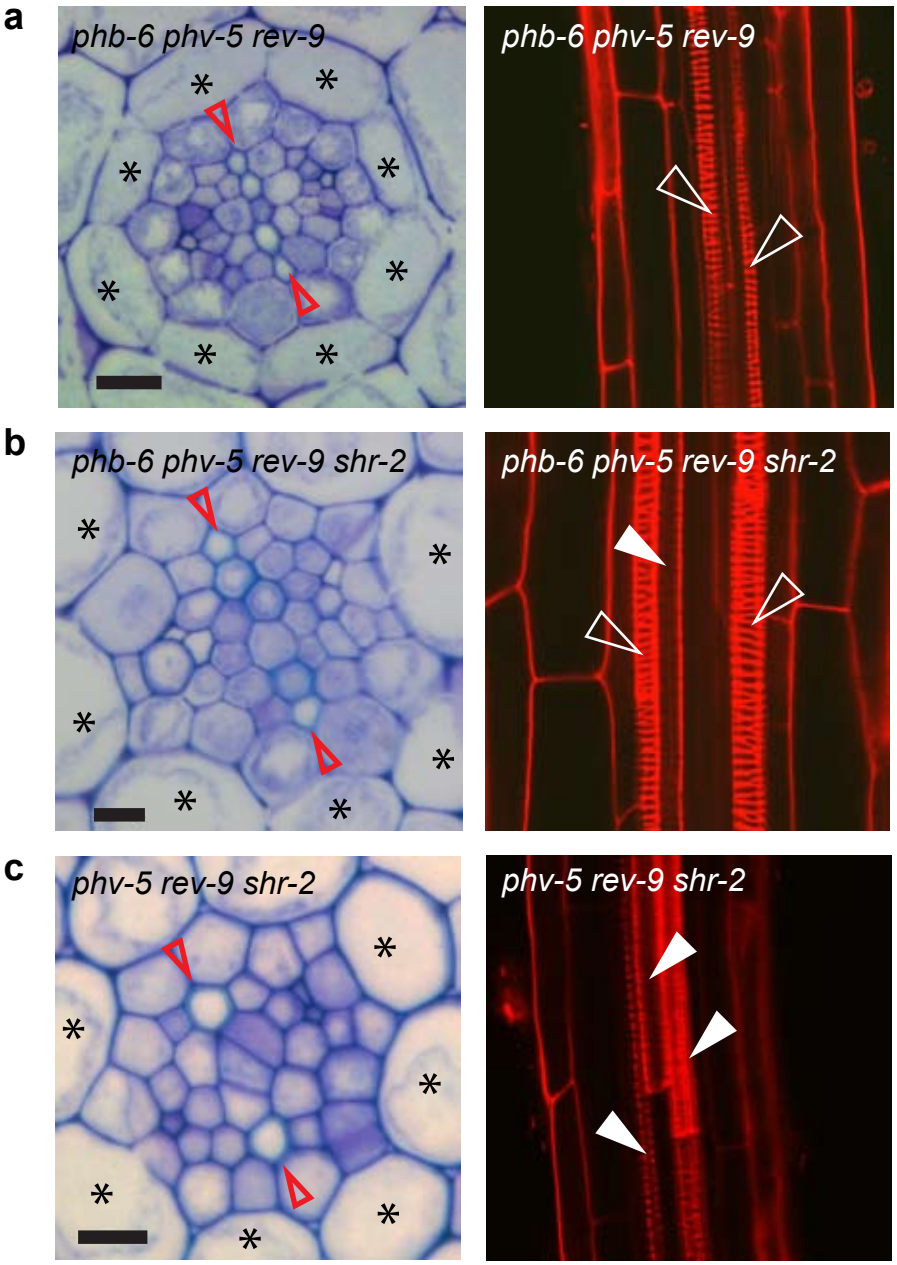

**Supplementary Figure 8. Ectopic metaxylem formation in the *hyl1* mutant.**

**a**, Summary of the xylem phenotypes of mutants of genes encoding components of the small RNA pathways. Only the mutant of *HYL1*, encoding a protein shown to be specifically involved in the miRNA mediated mRNA degradation [21, 22], formed ectopic metaxylem in the protoxylem position. **b**, Cross section and light micrograph of *hyl1-1*. Confocal laser scanning micrographs of *hyl1-1* and *ago1*. Asterisks mark endodermis, filled arrowheads indicate metaxylem, unfilled arrowheads indicate protoxylem. Scale bar is 10 μm.



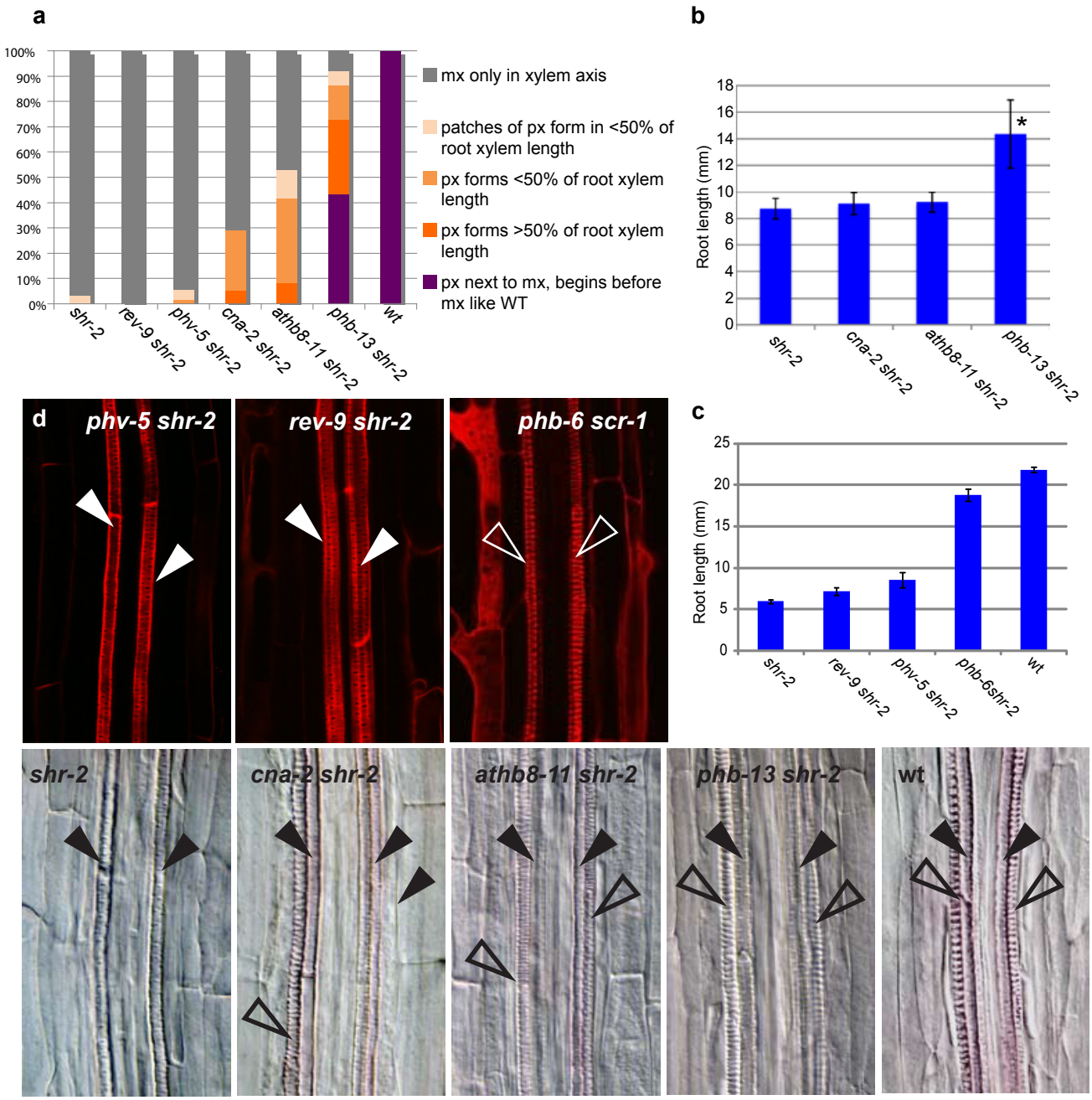

**Supplementary Figure 9. Messenger RNA levels of HD-ZIP III TFs are significantly elevated in *shr* and *scr* mutants.**

Expression ratios are shown in average log2 (fold change) extracted from the comparison between a mutant and wt. In this comparison, genome-wide transcript profiling data of 5 day-old-seedling roots from wild type, *shr-2*, and *scr-4* [14, 15] were normalized using the GCRMA method [42]. Error bars indicate standard deviation from the pairwise comparison.



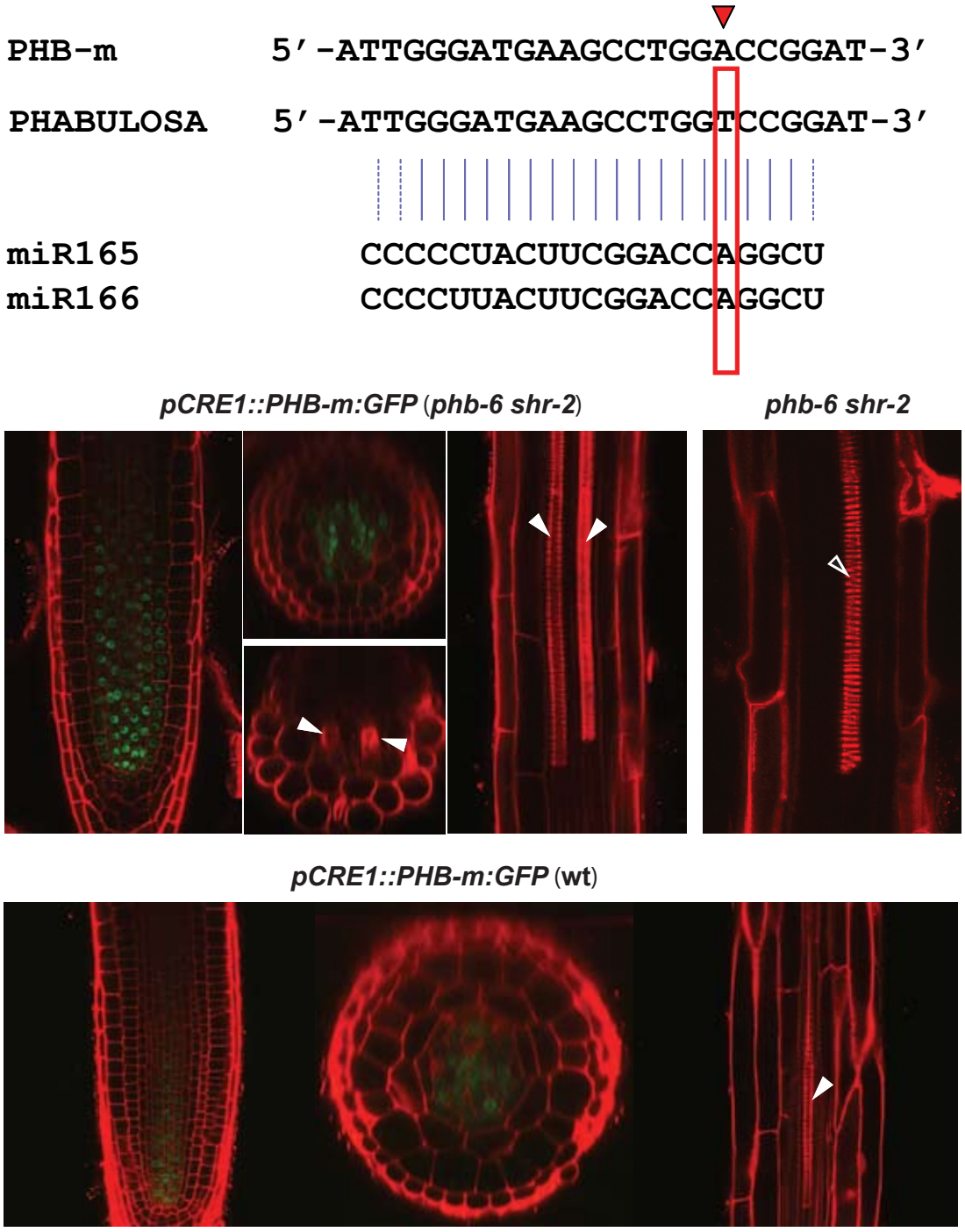

**Supplementary Figure 10. Down-regulation of *PHB* by SHR is required for protoxylem formation in the stele periphery.**

**a,** The triple mutant of *PHB*, *PHV*, and *REV* forms a similar xylem patterning and stele cell organization as the wild type root. **b,** The ectopic metaxylem formation and reduced stele cell number in the *shr* mutant is fully rescued in the *phb phv rev shr* mutant. **c,** Unlike the *phb phv rev shr* quadruple mutant, the *phv rev shr* triple mutant does not show rescue of the *shr* mutant phenotype. Ectopic metaxylem is formed in the stele periphery and the stele cell number is still reduced. Filled white arrowhead indicates metaxylem; unfilled white arrowhead indicates protoxylem; * marks the endodermis in **a** and the ground tissue in **b** and **c**; red arrowhead points at the xylem axis. Scale bar is 10 μm.



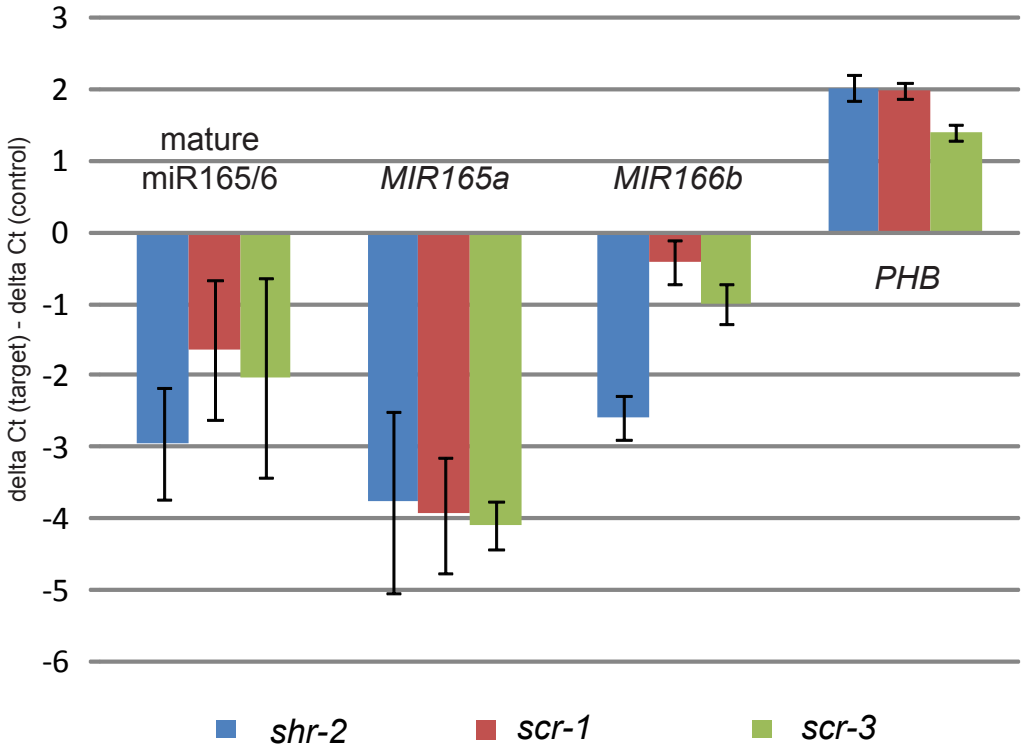

**Supplementary Figure S11. Loss of protoxylem in *shr-2* and *scr-1* can be recovered by loss of *PHB*, and to a lesser extent by loss of *ATHB8*, *PHV* and *CNA* but not by the loss of *REV*.**

**a,** Quantification of xylem cell types and patterns observed in the listed geneotypes. Protoxylem recovery most like wild type (wt) occurs in the double mutant of *SHR* and *PHB*; though *phv-5, cna-2*, or *athb8-11* in *shr-2* background can partially recover protoxylem, none of them could completely recover the wt xylem pattern. *shr-2 rev-9* never recovered protoxylem. **b,** Quantification of 5 day old root lengths for each of the genotypes listed. n=30~35. error bar=s.d. * denotes a p-value <0.001 (two-tailed t-test). **c,** Quantification of 10 day old root lenghts. n=18~30. error bar= S.E. **d,** Light or confocal microscope images showing a representative example of xylem observed in the double mutants of *HD-ZIP III* and *SHR/SCR*, in addition to images of *shr-2* and wt (Col-0) xylem patterns. Filled arrowheads indicate metaxylem (mx) and unfilled arrowheads protoxylem (px).

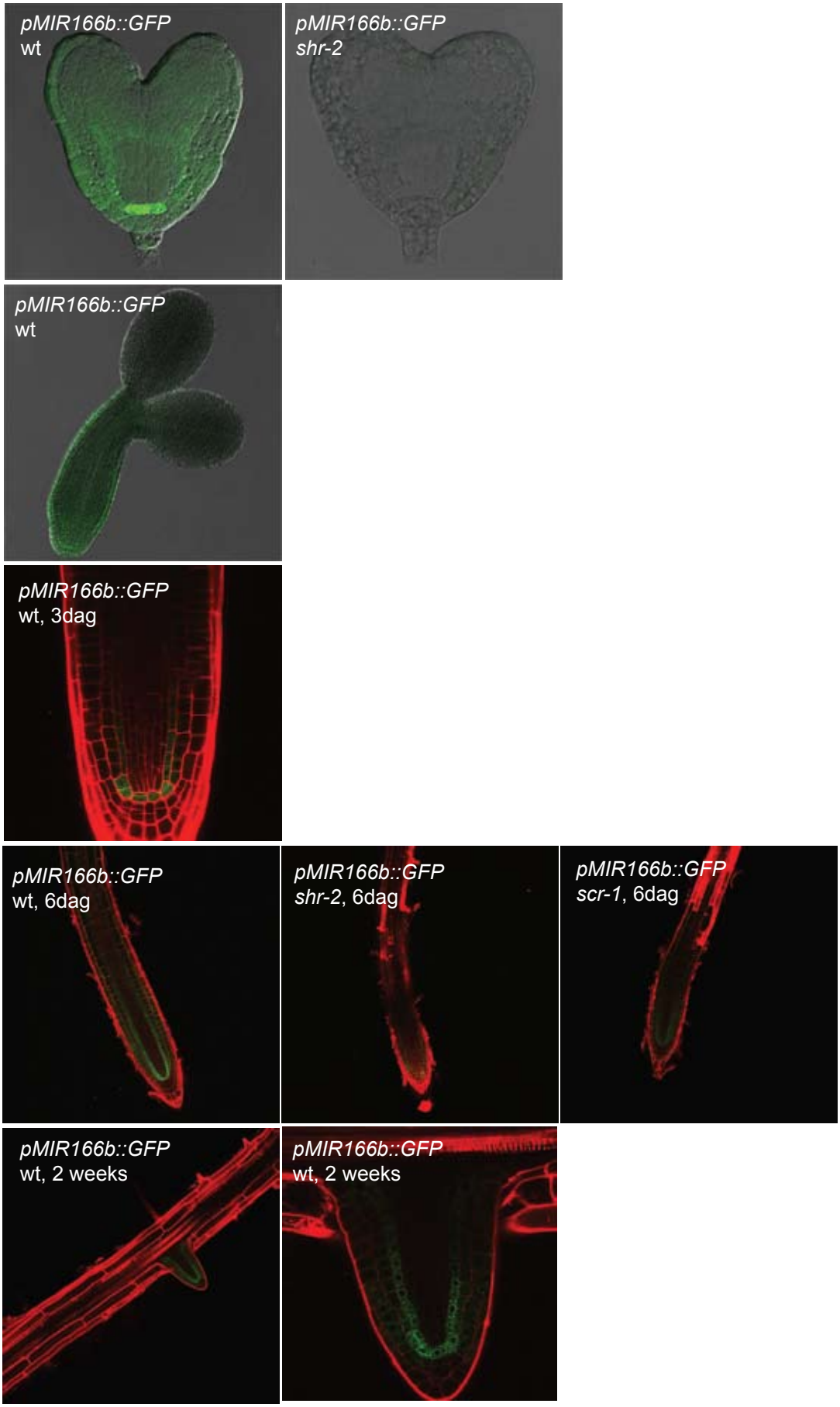



**Supplementary Figure 12. *PHB* with a silent mutation in the miR165/6 target sequence expressed in the stele is sufficient to form ectopic metaxylem in *phb shr* and wild type.**

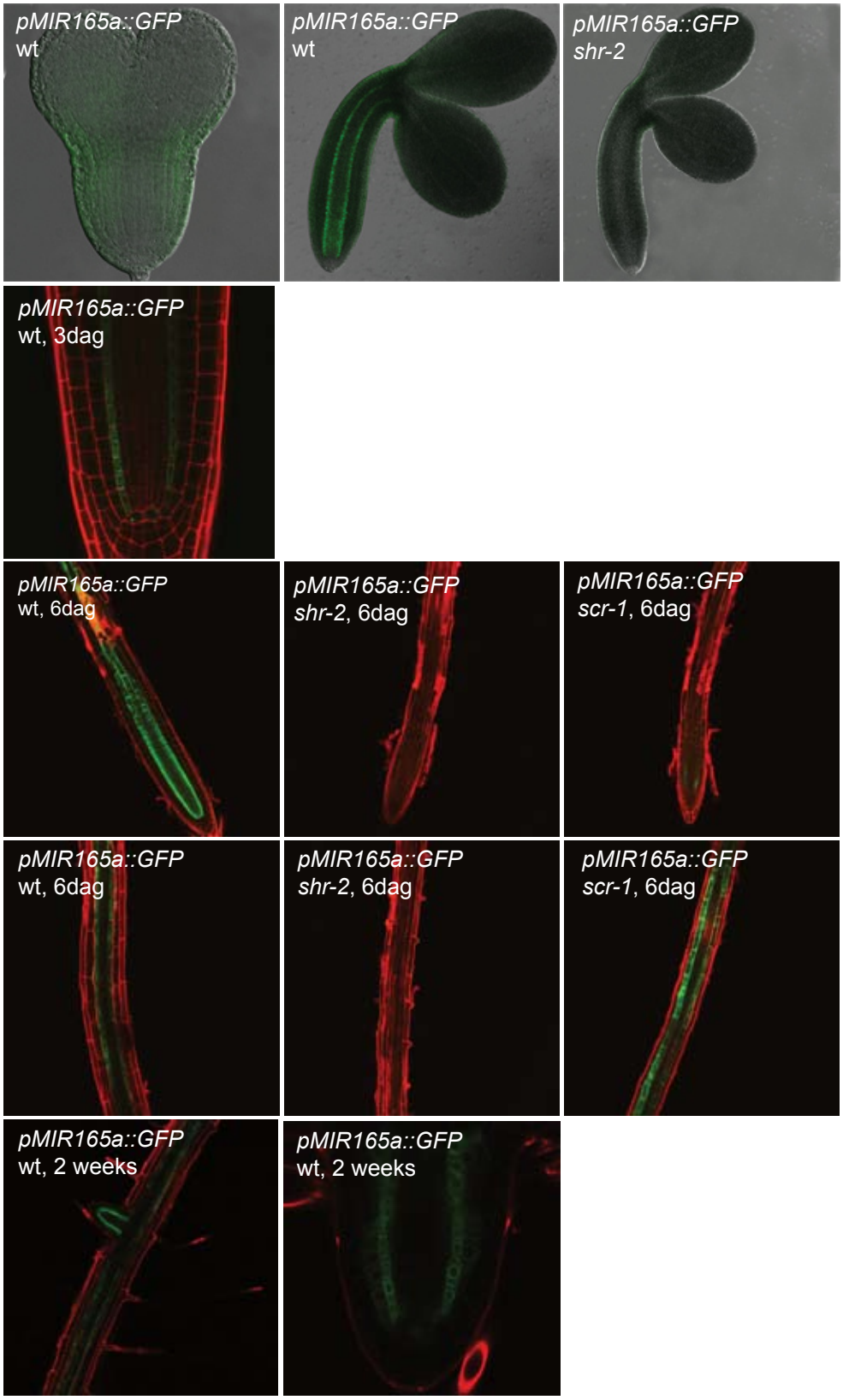
p*CRE1::PHB-m:GFP* was expressed in the double mutant of *SHR* and *PHB* and the wild type. In both genetic backgrounds the miR165/6 resistant *PHB* broadens its protein domains throughout the stele and causes ectopic metaxylem formation. GFP is sporadically in the ground tissue layer of *phb shr*, which is not observed with p*CRE1::erGFP* in *phb shr* (data not shown). Filled arrowheads indicate metaxylem; unfilled arrowhead marks protoxylem.



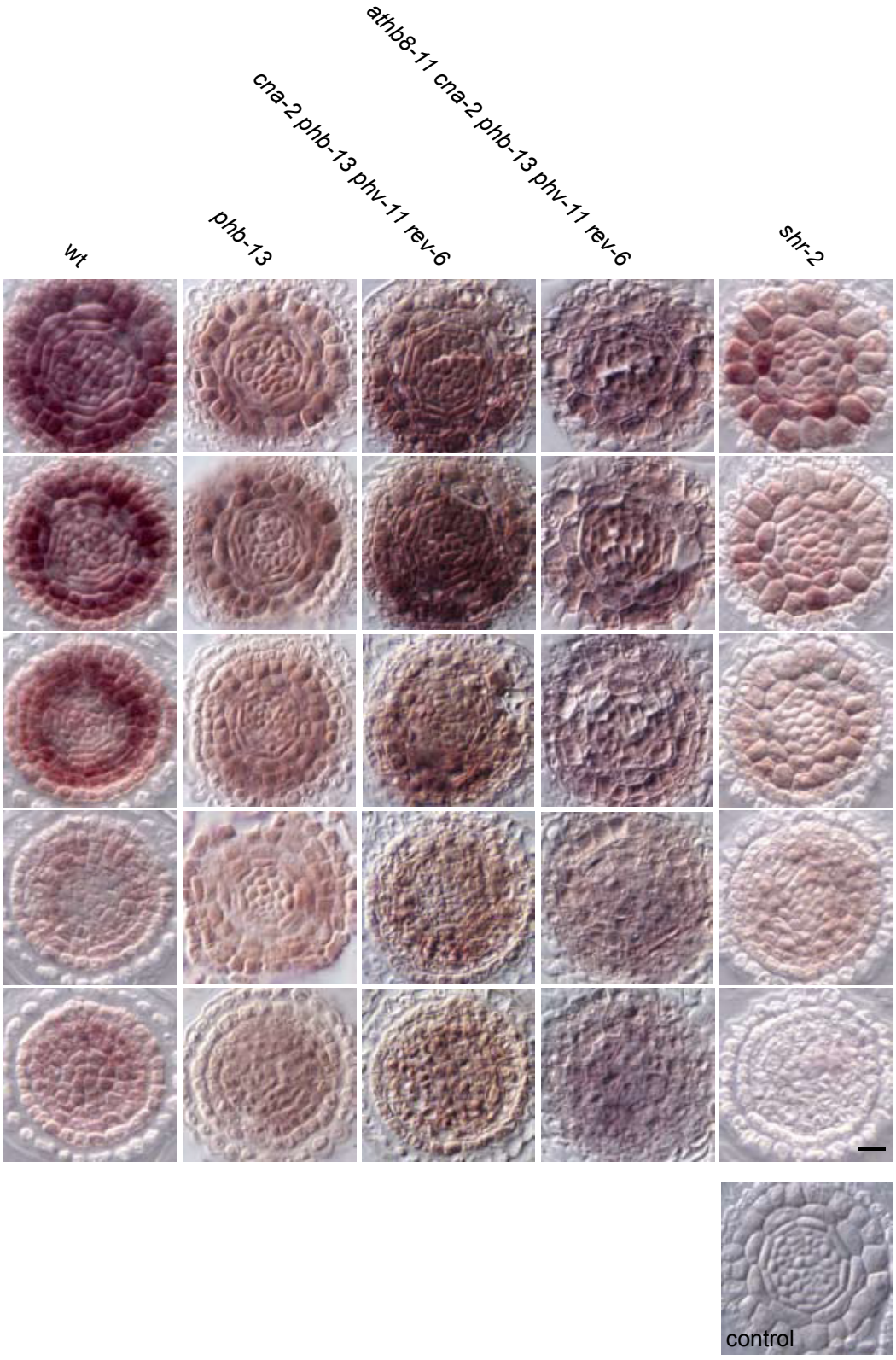

**Supplementary Figure 13. Levels of mature miR165/6 as well as *pri-MIR165a* and *pri-MIR166b* are reduced in the mutants of *SHR* and *SCR*. n=6. All data are presented as mean ± s.d.**




**Supplementary Figure 14_1. GFP expression patterns of *pMIR166b::GFP* in *shr*, *scr*, and wt roots.** (dag = days after germination)

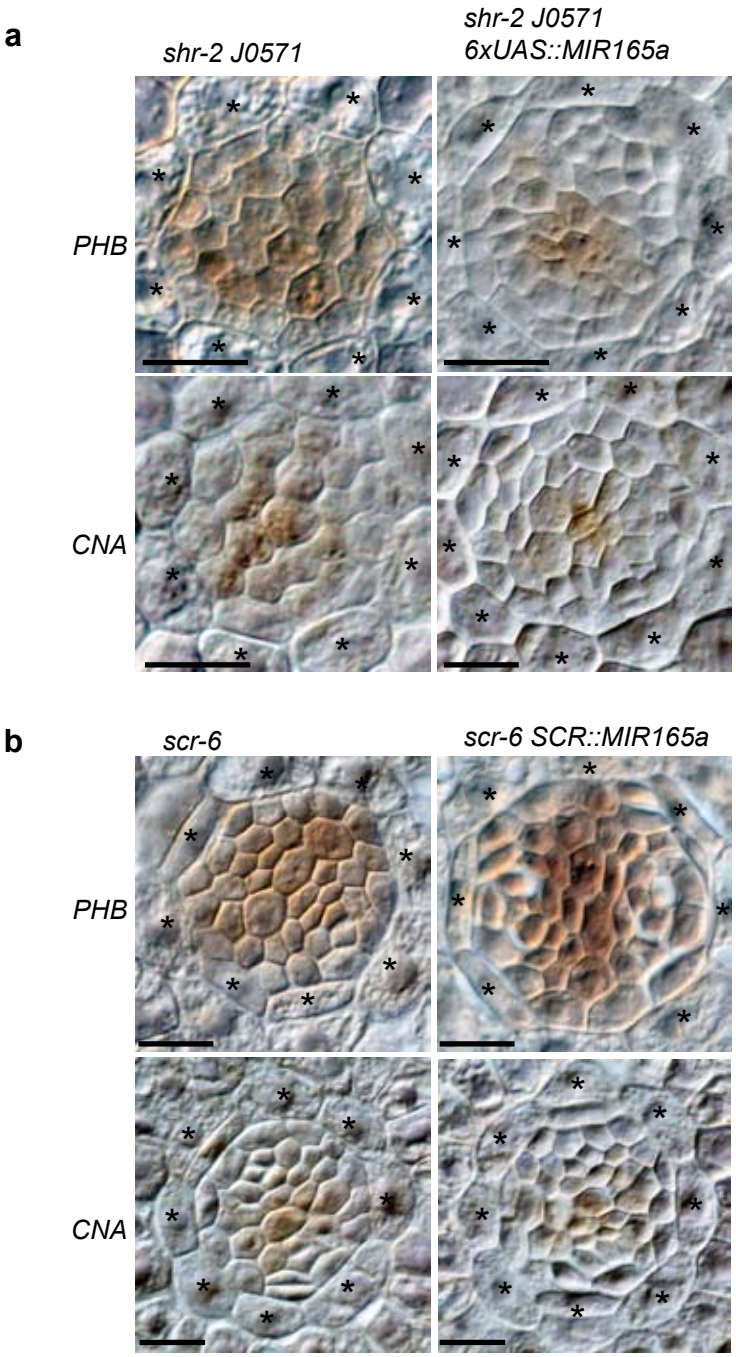



**Supplementary Figure 14_2. GFP expression patterns of *pMIR165a::GFP* in *shr*, *scr*, and wt roots.**



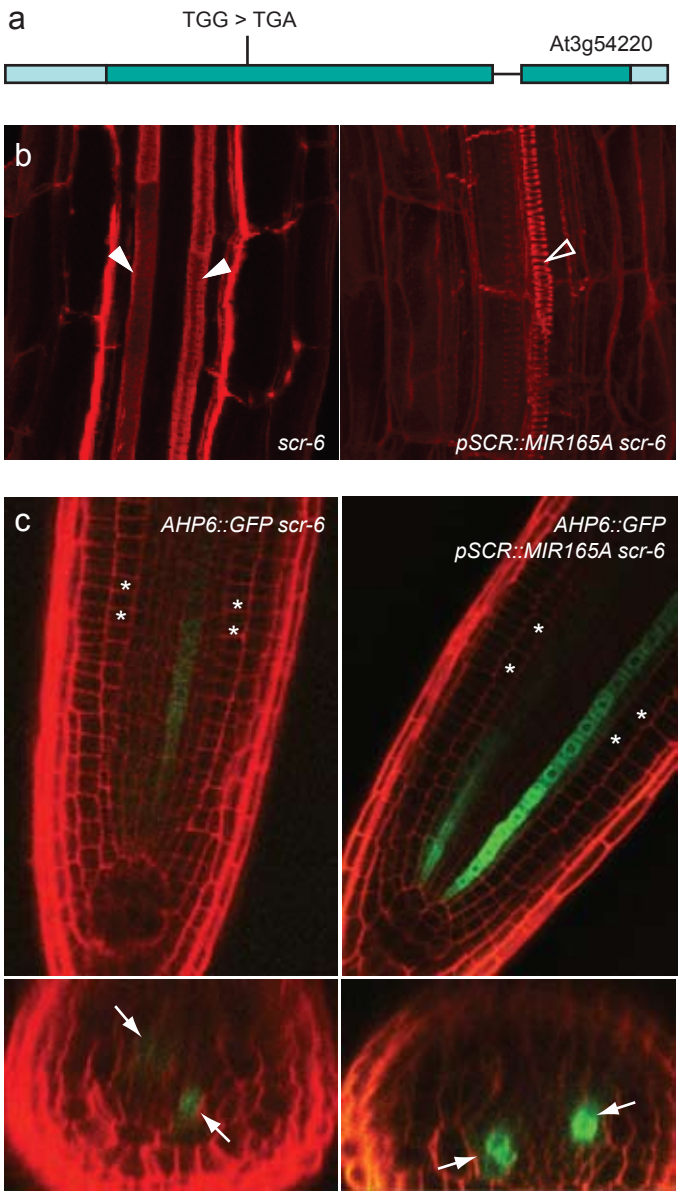

**Supplementary Figure 15. In situ hybridization with a miR166 specific LNA probe.**

Hybridization to cross sections of wt, *phb-13*, *cna-2 phb-13 phv-11 rev-6*, *athb8-11 cna-2 phb-13 phv-11 rev-6* and *shr-2*. Every or every second 7 µm section from the QC and up is shown. The negative control probe has three miss-matches in the complementary miR166 sequence. Scale bar represents 10 µm.



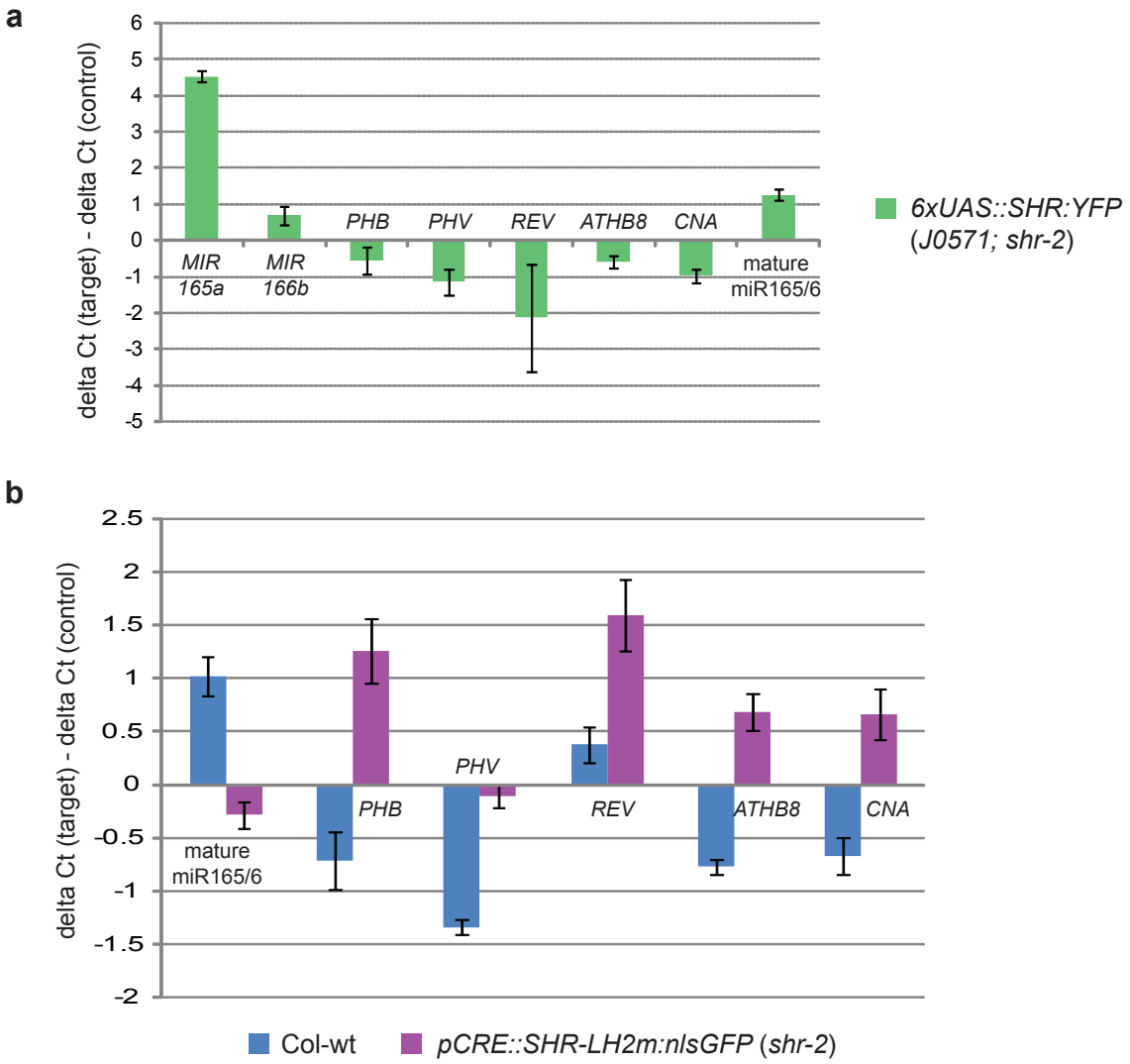

**Supplementary Figure 16. *PHB* and *CNA* mRNA localization domains are restricted within the vascular cylinder by ground tissue-driven *MIR165a* in *shr-2* and *scr-6.***

**a**, In situ hybridization of *PHB* and *CNA* antisense probes to cross-sections of *shr-2 J0571* and *shr-2 J0571 6xUAS::MIR165a* root meristems. **b**, *In situ* hybridization with *PHB* and *CNA* antisense probes to cross-sections of *scr-6* and *scr-6 SCR::MIR165a* root meristems. Asterisks mark the ground tissue. Scale bar is 10 μm.



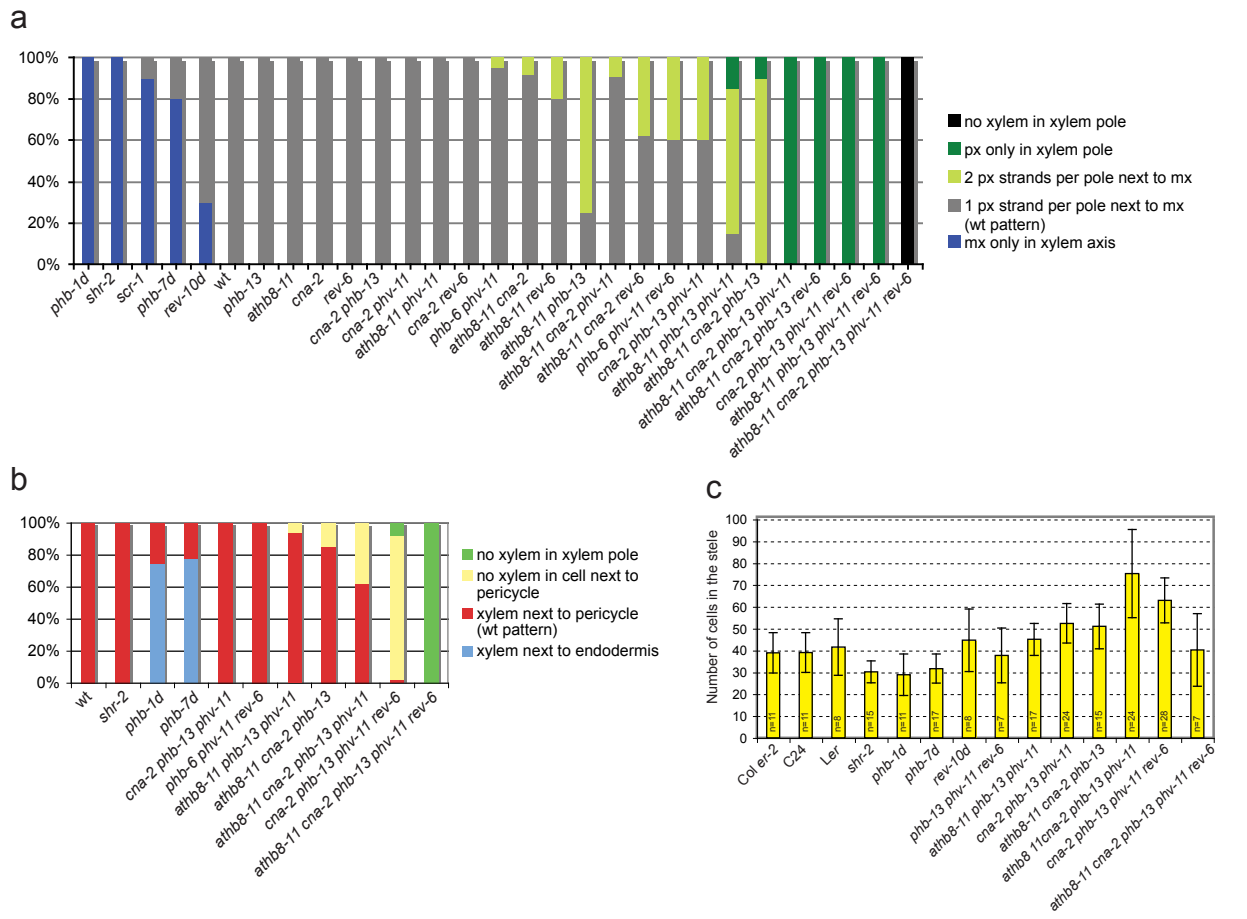

**Supplementary Figure 17. *MIR165a* under the ground tissue-specific promoter *pSCR* is sufficient to recover protoxylem in the novel *scr-6* allele.**

**a**, The mutation in *scr-6* is TGG to TGA creating a stop codon at position 426 of the *SCR* coding region. **b**,**c** Ectopic metaxylem (**b**) and reduced expression of the protoxylem marker *pAHP6*::GFP in *scr-6* (**c**) is restored when *pSCR::MIR165a* is introduced (in four out of 12 independent transgenic lines). Filled arrowheads indicate metaxylem, unfilled arrowheads point at protoxylem. Arrows indicate the xylem axis. Asterisks mark the ground tissue.



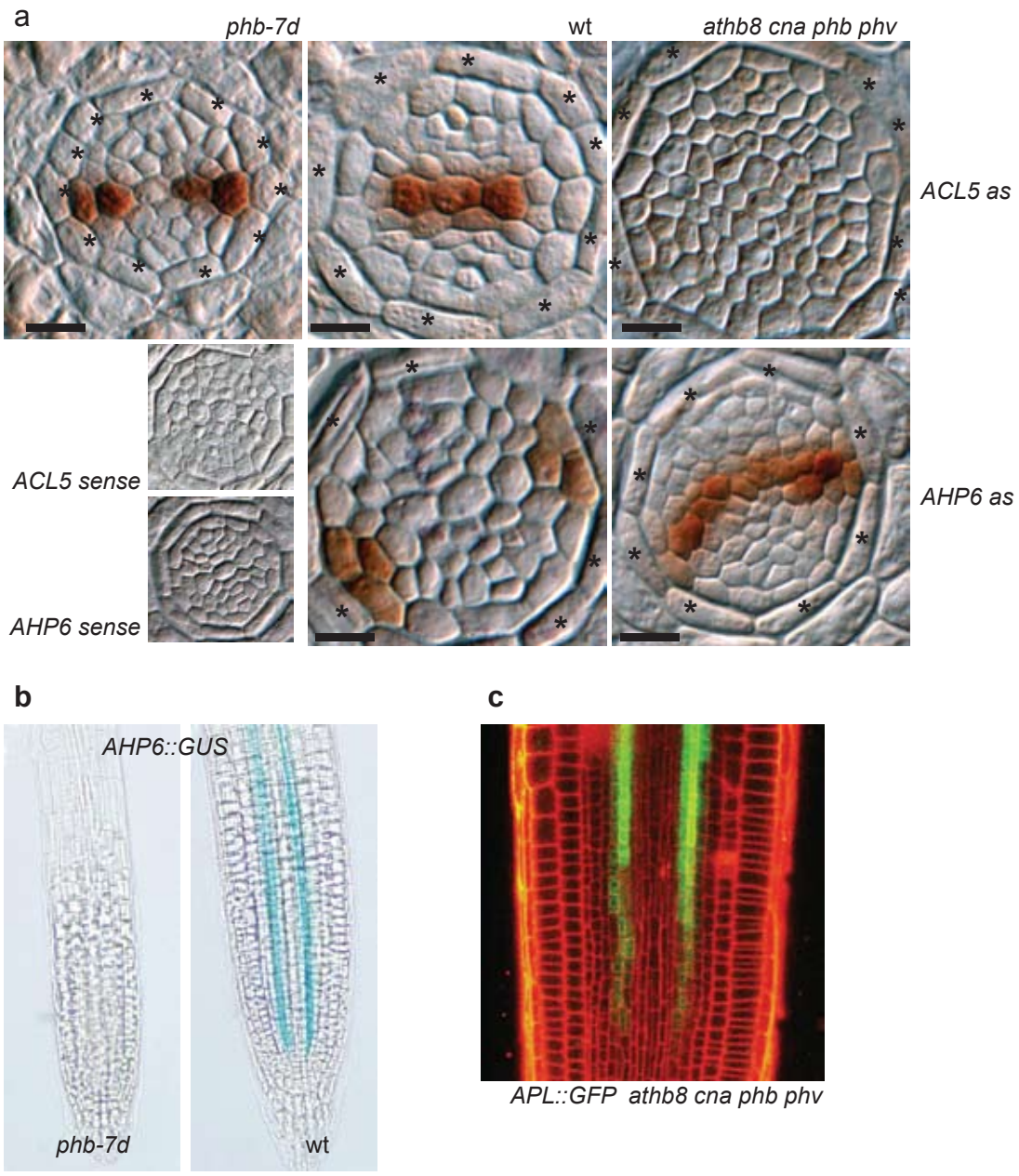

**Supplementary Figure 18. Xylem patterning requires SHR in the endodermis.**

**a**, *SHR* expressed specifically in the endodermis increases mature miR165/6 by activating the expression of *MIR165a* and *MIR166b*. This results in the suppression of all the *HD-ZIP III* TFs that are expressed in the stele. n=4. **b**, Non-mobile SHR expressed in the stele cannot activate *MIR165/6* expression, therefore failing in suppressing *HD-ZIP III* TFs. Roots from 7 day-old seedlings were used for the RNA analyses. n=4. Two biological replicates and three technical replicates were used in these experiments and compared against *J0571; shr-2* (**a**) and *shr-2* (**b**) as controls. All data are presented as mean ± s.d.




**Supplementary Figure 19. HD-Zip III TFs redundantly determine xylem patterning along the radial axis.**

**a,** Xylem cell types determined from fuchsin stained roots and presented per xylem pole. **b,** Xylem pattern determined in radially sectioned roots. **c,** Stele cell number as determined in root section ca 2 mm from the root tip, in fully differentiated tissues of 5 day old plants. No secondary cell divisions had taken place. Error bars show means ± s.d. n=7-28, given on respective bar. **d,** Xylem cell types in fuchsin stained *athb8-11 cna-2 phb-13* root. Filled arrowhead marks metaxylem, unfilled marks proto-xylem. **e,** Radial section of a *cna-2 phb-13 phv-11 rev-6* root. Black arrows indicate unlignified cells next to the pericycle along the xylem axis. **f,** Radial section of an *athb8-11 cna-2 phb-13 phv-11* root showing a triarch stele arrangement. **g,** Radial section through the meristem of a *athb8-11 cna-2 phb-13 phv-11 rev-6* root. White arrows indicate proto-sieve element cells, suggesting establishment of bilateral symmetry in the quintuple mutant. Scale bar is 10 μm.




**Supplementary Figure 20. Expression of metaxylem, protoxylem and phloem markers in gain- and loss-of-function *HD-ZIP III* mutants.**

**a**, *In situ* hybridization with a probe specific to the metaxylem marker *ACL5* [34] to cross sections of roots from 5 day old plants of *phb-7d*, wild type, *cna-2 athb8-11 phb-13 phv-11* and a control hybridization with an *ACL5* sense probe to a cross section of a wild type root. **b**, Expression of the protoxylem marker *AHP6::GUS* [35] in *phb-7d* and wild type and *in situ* hybridization with an *AHP6* specific probe to cross sections of wild type and *cna-2 athb8-11 phb-13 phv-11* roots. Control hybridization with an *AHP6* sense probe to a section of a wild type root. **c**, The phloem marker *APL::GFP* in *cna-2 athb8-11 phb-13 phv-11* indicates normal phloem development. See Supplementary Fig. 6 for *APL::GFP* pattern in *phb-7d*. Scale bar is 10 μm.

**Supplementary Table S1. List of cell type specific GFP markers used for comparing stele phenotypes in *shr-2* and *phb-7d***

| MARKER | Expression in wild type | Expression in *shr-2* | Expression in *phb-7d* | Reference |
|---|---|---|---|---|
| p*APL::GFP or pAPL::GUS* | Phloem. Expression starts right before the asymmetric cell division and continues to upper root | GFP expression starts in late maturation zone. Expression is much weaker than wild type | GFP expression starts in late maturation zone. Expression is much weaker than wild type | 43 |
| p*AT2G18380::GFP* | Phloem from initials | Wild type expression | Wild type expression, or spreading in vascular initials. | 27 |
| p*SUC2::GFP* | Two companion cells of each phloem pole starting in the late maturation zone | GFP expression is delayed, weaker, and frequently only in one pole | GFP expression is delayed, weaker, and frequently only in one pole | 41 |
| p*AT2G22850::GFP* | Phloem-pole pericycle | Wild type expression | Wild type expression | 27 |
| J0121 | Xylem-pole pericycle | Wild type expression | Expression is delayed relative xylem differentiation | http://www.plantsci.cam.ac.uk/Haseloff/Home.html |
| J3612 | From pericycle initials and up, distally to the meristem predominantly in phloem pole pericycle cells. | Delayed expression | Severely weak and delayed expression | http://www.plantsci.cam.ac.uk/Haseloff/Home.html |
| p*AT3G25710::GFP* | Protoxylem and metaxylem precursors prior to cell elongation. GFP is much weaker in metaxylem than in protoxylem precursors. | Very weak GFP in early xylem | Very weak GFP in early xylem | 27 |
| p*AHP6::GFP* | Xylem pole pericycle and protoxylem | no expression | No expression in primary root, weakly in emerging lateral roots | 35 |

**Supplementary Table 2. Expression of genes involved in mi/si-RNA regulatory pathways in *shr* and *scr* mutant roots.**

| locus | affy ID | gene names | *shr-2* | wild type | *scr-4* | Q value (*shr* vs. wt) | Q value (*scr* vs. wt) |
|---|---|---|---|---|---|---|---|
| **AT1G48410** | 262246_at | AGO1 | 6.01 | 6.73 | 2.52 | 0.650 | 0.0006 |
| **AT5G43810** | 249115_at | AGO10/ZLL | 1.46 | 1.16 | 1.08 | 0.100 | 0.088 |
| **AT1G31280** | 262548_at | AGO2 | 0.68 | 0.70 | 0.78 | 0.578 | 0.011 |
| **AT2G27040** | 266314_at | AGO4 | 1.63 | 2.16 | 1.20 | 0.220 | 0.001 |
| **AT2G27880** | 264066_at | AGO5 | 0.62 | 0.65 | 0.74 | 0.540 | 0.007 |
| **AT1G69440** | 256293_at | AGO7 | 0.66 | 0.57 | 0.81 | 0.015 | 1.06E-07 |
| **AT5G21150** | 246025_at | AGO-LIKE | 0.44 | 0.45 | 0.66 | 0.594 | 1.84E-11 |
| **AT1G31290** | 262548_at | AGO-LIKE | 0.68 | 0.70 | 0.78 | 0.578 | 0.011 |
| **AT1G01040** | 261584_at | DCL1 | 0.88 | 0.80 | 0.83 | 0.174 | 0.014 |
| **AT3G03300** | 258863_at | DCL2 | 0.94 | 0.92 | 0.93 | 0.668 | 0.152 |
| **AT3G43920** | 252716_at | DCL3 | 0.64 | 0.63 | 0.75 | 0.616 | 1.32E-05 |
| **AT5G20320** | 246117_at | DCL4 | 1.13 | 0.92 | 0.91 | 0.005 | 0.158 |
| **AT1G45230** | 245797_at | DICER-LIKE | 0.68 | 0.66 | 0.75 | 0.664 | 1.44E-05 |
| **AT3G62800** | 251233_at | DRB4 | 1.38 | 1.22 | 1.09 | 0.144 | 0.045 |
| **AT4G20910** | 254449_at | HEN1 | 0.70 | 0.75 | 0.78 | 0.021 | 0.055 |
| **AT2G06990** | 266501_at | HEN2 | 1.96 | 1.89 | 1.16 | 0.589 | 0.0057 |
| **AT1G09700** | 264677_at | HYL1 | 0.41 | 0.43 | 0.66 | 0.551 | 6.30E-07 |
| **AT3G49500** | 252261_at | RDR6 | 1.24 | 1.31 | 1.14 | 0.598 | 0.011 |
| **AT5G23570** | 249843_at | SGS3 | 0.48 | 0.48 | 0.90 | 0.662 | 3.35E-09 |

## Supplementary Online Methods

### Growth conditions

Seedlings were grown vertically on Petri plates containing 0.5x Murashige and Skoog salt mixture and 1% sucrose, pH 5.8, in 0.8% agar, at a light regime of 16 h light/ 8 h darkness at 22°C.

### Root expression patterns of downstream genes of SHR and SCR

CEL files of root transcript profiling from wild type, *shr-2* [15], and *scr-4* [14] were normalized by GCRMA [42] in R. Differentially expressed genes in *shr-2* and *scr-4* were identified using the LIMMA package [44] in R. Those with a corrected p-value below 0.05 were selected to visualize their expression patterns in wt roots [27,45-47]. Mature endodermis data that were obtained with cell sorting-microarray technique [48] using the E30 marker line [27] were added into the published root expression data [27,45-47]. Three biological replicate data sets are available in the GEO database (http://www.ncbi.nlm.nih.gov/geo/) under the accession number: Series GSE16469.

### ChIP-qPCR

500µl of Arabidopsis seeds were sterilized and cold-treated for two days. They were germinated and grown vertically on MS agar media covered with sterile nylon mesh (Sefar U.S.A.; cat. 03-100/47). Roots were harvested 6 days after germination and processed for ChIP following Cui et al., 2007 [14].

Proteins and DNA were cross-linked by fixing the roots in 1% formaldehyde for 10 minutes on ice. After stopping the fixation by treating the roots with 2.5M glycine and washing, nuclei were isolated. Cross-linked DNA/protein complexes in the nuclei were fragmented by sonication with a Branson Sonifier 250D. This fragmented chromatin prep was divided into two groups. One group was treated with anti-GFP antibody (ab290, Abcam) overnight and the other was not treated but processed following the same procedure as the one treated with anti-GFP antibody. Chromatin preps bound to GFP antibodies were isolated by incubating with Protein A agarose beads (Upstate, Cat#16-157) and subsequently release from the beads into the elution buffer. Proteins cross-linked to DNA were removed by incubating the chromatin prep

with proteinase K. DNAs were further cleaned by extraction and precipitation and dissolved in TE buffer prior to quantitative real-time PCR.

For quantitative real-time PCR, the PCR mixture with Power SYBR Green PCR master mix (Applied Biosystems), 0.35μM of forward and reverse primers, and ChIP DNA in 10mM Tris, pH 8.0 was amplified in an ABI 7900HT (Applied Biosystems U.S.A). To identify enrichment of the regions bound by SHR, ChIP DNA without antibody treatment was used as a control. 18S forward- and reverse primers were used as the internal control for comparing the amount of template DNAs. MGP forward- and reverse primers were used as the positive control since the region amplified by this primer pair was shown to be bound by SHR in Cui *et al.* (2007) [14]. Real time PCR started with incubation at 95°C for 10 min and was followed by 45 cycles of incubations at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Fold changes were calculated assuming the constant efficiency to be equal to 2.

The list of primers used for ChIP real time qPCR

| Distance from transcription start site (kb) | Primer name | sequence |
|---|---|---|
| -0.8 | miR165a-1F | ATTCCCCACGCCAGAAAGAA |
| | miR165a-1R | TGAAACCACAGGCTGCCAAA |
| -1.4 | miR165a-2F | AAATGGATGCCATTATGGTGAAGAG |
| | miR165a-2R | TGATTACTGACAAACGGAGAAGACG |
| -2.7 | miR165a-3F | TGAAACTTTTTGGTGGTAATGAGCC |
| | miR165a-3R | GCTACGACCGCAATCTTAGTGTTTA |
| -3.6 | miR165a-4F | ATTCGCCCACCTTTTATTCATTAGG |
| | miR165a-4R | CCCAAGCTGAAACAAATGACACG |
| -0.2 | miR165b-1F | GTTTGATTATCGAATAGCCACACCA |
| | miR165b-1R | TTCCGAAACCCTAGTCTTGTCTTCT |
| -1.1 | miR165b-2F | AATGGCTTTTTGGCAAGACGG |
| | miR165b-2R | TTCTTTACCCGTCCCGTGATTT |
| -2.6 | miR165b-3F | TGGATAGCATCAGCTTGAAGAGCAT |
| | miR165b-3R | TGGAACTTCACGCCACTGTCTAA |
| -3.2 | miR165b-4F | GGAGCTATTGCCTTGTCCTTATTG |
| | miR165b-4R | CACATCCTGGGAATTTGTAGTTTGA |
| -0.8 | miR166a-1-1F | ATCTCTTTCACCGGATGTTCTTACGC |
| | miR166a-1-1R | TTGCAAGTGGAAGGAGACTAACT |

| | | |
|---|---|---|
| -1.8 | miR166a-2F | AAACACATGATGACTTCATCTCCGT |
| | miR166a-2R | CCCATTTCCCTTGGTGACTCTT |
| -2.4 | miR166a-2-1F | TAACTAGTCATATTTCCAGCTGGC |
| | miR166a-2-1R | TCGAATATTGGACAGGATTTTAC |
| -3.6 | miR166a-4-1F | TAAGTAACGTCCTGCCAAAC |
| | miR166a-4-1R | CTCTCTACATTAACTACACGATTGG |
| -4.6 | miR166a-5F | GAGGGGTAAAAAAAAGAGTGATGGG |
| | miR166a-5R | TCCACTATCCTACTCCTTCCTCACC |
| -8.5 | miR166a-7F | CTGGTCGATGTGAAAATGGAAATGGA |
| | miR166a-7R | CGTCACACATCCTGACTTCTGAACG |
| -0.5 | miR166b-1F | TTGGGCAGATCAATAACTCAACCAC |
| | miR166b-1R | CAGTTTTCCTTTCACGCCCAT |
| -1.6 | miR166b-2F | ACTCCACGCTTACACTAACATACGC |
| | miR166b-2R | CGCGACATGTGCTGAAACATTT |
| -3.0 | miR166b-3-1F | AAAAGCCCAGTTAGACCATCTGAACC |
| | miR166b-3-1R | TGCGGTCACAATGAAAACACTTTG |
| -3.6 | miR166b-4F | GTAGGGTCCATGATAAGATGCCAAT |
| | miR166b-4R | TGAATCCGAAGGTTCCTCAAGTAGT |
| -4.7 | miR166b-seq3 | CGGCCATTTGTGAACTATGG |
| | miR166b-5R | AATGGCAATGAGTCAGTGGTTGA |
| -5.7 | miR166b-6F | AGATTTCATTTGTCGTTACTCGCACG |
| | miR166b-6R | CCGTGTAACGAAATTAAACGCGTAA |
| -6.5 | miR166b-7F | TGCTTGTGTAGGGCCTCGGATTTCT |
| | miR166b-7R | GTTCGCCCGTTTGATTGTCAACC |
| -5.0 | miR166b-8F | GAAAACGAAGTCAGTGTAGT |
| | miR166b-8R | GAAGCCGCCATAGTTCACAAATGG |
| -4.4 | miR166b-9F | TGTGTATGAAAATTGCGTCCGACAA |
| | miR166b-9R | GGCTCTATAGTCTTATGTAT |
| -0.2 | miR166c-1F | ACGTCTTCCCGTTATTGAAAGTCTC |
| | miR166c-1R | CACAAATGACCACAACCTCAAGATT |
| +1.0 | miR166c-2F | TTACGTGCCTTTTATTTGCTCTTCG |
| | miR166c-2R | GCTATGGGAAAGTTTTGCTCAACTA |
| -0.2 | miR166D-1F | TAGAAACCATGGTACTTGTCTCCAA |
| | miR166D-1R | GGAGCAAATGACCACAAGACAAA |
| -0.5 | miR166e-1F | AGATGGGAGGCATGTGATTGTCAC |
| | miR166e-1R | CACATGTAAATCCGTGAGTCGTCA |
| -1.4 | miR166e-2F | CAACGTAATGCTTGGTGTTGGA |
| | miR166e-2R | CGGAATATCAACTTCGCAACCTTTA |

| | | |
|---|---|---|
| -2.2 | miR166e-3F | GTAAACGAACAAGCCTTCTTGGAA |
| | miR166e-3R | CCAATCTAAACACGATGCTTGTGTA |
| -0.2 | miR166f-1F | CGTTTTTGCTTTTTGGTGAGTAGC |
| | miR166f-1R | TGGCTCAGAAAGACAGAGAGAGAAG |
| -0.7 | miR166G-1F | ATAAATCGAGAGGAAGGTACTTGTC |
| | miR166G-1R | GGATTGAGTGTTTGTCTTTAGTACC |
| -1.1 | miR166G-2F | TGGTTTGTTATGACGTGTTCTTCGGT |
| | miR166G-2R | ATATTTTGAATATCATCCCACTT |
| -1.7 | miR166G-3F | ACCCCACTTGTGACATTGTTTATGGC |
| | miR166G-3R | CGCACCCAAAGTACACAACACCAA |
| Positive control | MGP-F | GTATCTGTGTTATAAGTAACAATG |
| | MGP-R | GGTTAGATCCATACCATAGCAT |
| Internal control | 18S-F | TACCGTCCTAGTCTCAACCA |
| | 18S-R | AACATCTAAGGGCATCACAG |

**Measurement of RNA expression**

Root tips from 6 or 7 day-old seedlings were harvested and total RNAs including small RNA were extracted with miRNeasy Mini Kit (Quiagen). To measure the expression level of pri-miRNA and other mRNAs, cDNA was synthesized using SuperScript III first strand synthesis system for RT-PCR (Invitrogen). To measure mature miRNAs, a polyadenylation reaction was performed prior to the reverse transcription following the method by Shi and Chiang [39]. 500ng of total RNA including small RNAs was incubated with 0.2 units of *E.coli* Poly(A) Polymerase and ATP (Ambion, #1350) at 37°C for 1 hour. cDNA from polyadenylated RNA was synthesized using SuperScript III first strand synthesis system for RT-PCR (Invitrogen) and polyT-adaptor as a primer. Real time PCR was performed on an ABI 7900HT (Applied Biosystems) following the same procedure as ChIP qPCR with 40 cycles at 56-58°C as an annealing temperature. To amplify the mature miRNA165/166, the primer pair of qmiR165 and reverse-miR was used. To find good internal controls, constitutively expressed and least variable genes were selected from root transcript profiling of wild type, *shr-2* and *scr-4* plants [14,15]. Two genes *at4g39910* (ubi1) and *at1g08190* (ubi2) were selected and used as internal controls, in addition to *UBQ10* [49]. Quantitative RT-PCR for detecting expression level differences in wt and *phb-7d* was performed as previously described [35], using primers (PHB.F) and (PHB.R).

Primer sequence for real time RT PCR

| Primer name | Sequence |
|---|---|
| SCR-qF | GGAATGTATTAGCGGTTGGA |
| SCR-qR | AGAACGAGGCGTCCAAGC |
| PHB-qF | GCTAACAACCCAGCAGGACTCCT |
| PHB-qR | TAAGCTCGATCGTCCCACCGTT |
| PHB.F | CTACTCCGAACGGTGCATCTG |
| PHB.R | GTTACAGCCATTTGGGTCGGC |
| PHV-qF | GCTAATCTTCTCTCGATTGCGGAGGA |
| PHV-qR | GCTCGATAGTACCACCATTTCCAGTG |
| REV-qF | GAATGATAGGTTGCAGAAGCAGGTTT |
| REV-qR | ATTGCATCTTTGCGAAATGGCA |
| ATHB8-qF | AACACCACTTGACCCCTCAACATCAG |
| ATHB8-qR | CACGCAACCAACAAGGCTTATCC |
| ATHB15-qF | ATTGGCATCTCAAAATCCTCAGAGA |
| ATHB15-qR | GGCAACACGTTCATAACTTCAACAGC |
| 165A-qFn | GATCGATTATCATGAGGGTTAAGC |
| 165A-qRn | CTATAATATCCTCGATCCAGACAAC |
| 166A-qFn | GGGGCTTTCTCTTTTGAGG |
| 166A-qRn | CGAAAGAGATCCAACATGAATAG |
| 166B-qFn | GATTTTTCTTTTGAGGGGACTGTTG |
| 166B-qRn2 | CTGAATGTATTCAAATGAGATTGTATTAG |
| 166C-qFn | GCGATTTAGTGTTGAGAGGATTG |
| 166C-qRn | GTTCTTCCAAATTAATTCGAGTG |
| 166D-qFn | GGTTGAGAGGAATATTGTCTGG |
| 166D-qRn | TTTAGGGATTTCACTCTTTAAAATG |
| 166E-qFn | GAGGGGAATGTTGTCTGG |
| 166E-qRn2 | GAAGAGACATATATATATAATCAAATATAGATC |
| ubi2-F | CGATTGGTCAAAATCGTAACAG |
| ubi2-R | CTTCATGGCTCAGGCAGAC |
| ubi1-F | AGCTCCACAGAGACGCTTCAC |
| ubi1-R | GCTTCTTGTAGCGGCCTAGC |
| UBQ10.F | CACACTCCACTTGGTCTTGCGT |
| UBQ10.R | TGGTCTTTCCGGTGAGAGTCTTCA |
| qmiR165 | CGGACCAGGCTTCATCCC |
| polyT-adaptor | GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTVN |
| reverse-miR | GCGAGCACAGAATTAATACGAC |

### *In situ* hybridization

For riboprobe *in situ* hybridization, tissue samples were prepared from 4 to 5 day-old roots; sectioning, preparation of the probes and *in situ* hybridization were performed as described [50]. The *AHP6* sense and antisense probe templates were amplified as described [50]. The antisense and sense template for the *ACL5* probe was amplified with primers described in Birnbaum *et al.* (2003) [45].

Primer sequences for riboprobe amplification

| Primer name | sequence |
|---|---|
| PHB.s. F | GgatcctaatacgactcactatagggaggCTTTGGTAGTGGCGTGCTTT |
| PHB.s.R | AATGTGAAAACCGGTGAAGC |
| PHB.as.F | CTTTGGTAGTGGCGTGCTTT |
| PHB.as.R | GgatcctaatacgactcactatagggaggAATGTGAAAACCGGTGAAGC |
| AtHB15.s.F | GgatcctaatacgactcactatagggaggCCAGACAAGCTAATGGGTCTA |
| AtHB15.s.R | TTCATTGCCGTCACTGTTTGA |
| AtHB15.as.F | CCAGACAAGCTAATGGGTCTA |
| AtHB15.as.R | GgatcctaatacgactcactatagggaggTTCATTGCCGTCACTGTTTGA |
| AtHB8.s.F | GgatcctaatacgactcactatagggaggCCCACAAAGATGATGATGACTT |
| AtHB8.s.R | AGATCAGTTCCATGAGGTTTG |
| AtHB8.as.F | CCCACAAAGATGATGATGACTTC |
| AtHB8.as.R | GgatcctaatacgactcactatagggaggAGATCAGTTCCATGAGGTTTG |
| PHV.s.F | GgatcctaatacgactcactatagggaggCTGGCTCCCAATACGGTAGC |
| PHV.s.R | CAATATGGCATCACTATGGTC |
| PHV.as.F | CTGGCTCCCAATACGGTAGC |
| PHV.as.R | GgatcctaatacgactcactatagggaggCAATATGGCATCACTATGGTC |
| REV.s.F | GgatcctaatacgactcactatagggaggCTACAAAGCATTTGAATAATAT |
| REV.s.R | AACATGAACACTGGCTGTGG |
| REV.as.F | CTACAAAGCATTTGAATAATAT |
| REV.as.R | GgatcctaatacgactcactatagggaggAACATGAACACTGGCTGTGG |

For LNA probe *in situ* hybridization, tissue fixation and hybridization were performed as described [19], except that the tissues were embedded in Histowax (Histolab Products AB, Goteborg, Sweden), sectioned 7 μm thick and mounted on Superfrost PLUS slides (Fisher Scientific). LNA probes with complementary sequences to miR165 or miR166 were synthesized and 5' digoxigenin-labeled by Exiqon (Vedbaek, Denmark). The miRNAs share 21 of 22 nt, and the probes therefore likely cross-hybridize, and

consistently the two probes gave similar results. As control probes we used the miR166 LNA probe sequence with three nucleotide changes, 5'-gg**C**gaatg**T**agc**G**tggtccga-3', as well as the murine miR124 synthesized and 5' digoxigenin-labeled by Exiqon. 3.125 picomoles of each LNA probe were used per slide, and the probes were hybridized overnight at 50°C. Sections were mounted in 50% glycerol, analyzed in a Leica Leitz DM RXE light microscope at 40X magnification and imaged using differential interference contrast (DIC) settings and a Leica Microsystems DFC-490 digital camera. Contrast and brightness were adjusted equally for all images within one experiment in Adobe Photoshop CS3.

**DNA manipulation and transgenics**

1. *UAS::SHR:YFP* and *UAS::SCR:YFP*

SHR and SCR cDNA without stop codons were cloned into pDONR221 and eYFP (Clontech) was cloned into pDONR P2R_P3 by BP recombination. 6xUAS cloned into pDONR P4_P1R, SHR/SCR into pDONR221, and eYFP into pDONR P2R_P3 were recombined into dpGreen-BarT, a modified vector of dpGreen-BAR by inserting a 19S terminator outside the attR3. 6xUAS::SHR:YFP and 6xUAS::SCR:YFP in *Agrobacterium* GV3101 with pSOUP [51] were transformed into *J0571;shr-2*.

2. *CRE1::SHRΔNLELDV:nlsGFP*

*CRE1* promoter was amplified from Columbia genomic DNA using primers (forward: 5'-GGGGACAACTTTGTATAGAAAAGTTGTTCCTAGATTTTCTCACACACCA-3' and reverse: 5'-GGGGACTGCTTTTTTGTACAAACTTGATCTGAGCTACAACAATAGAGAA -3') and cloned into pDONR P4_P1R by BP recombination. *SHRΔNLELDV* was amplified from a plasmid containing *SHRΔNLELDV* [18] with primers (forward 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGGATACTCTCTTTAGACT AGTCA-3'and reverse 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTACGTTGGCCGCCACGCACTAGC CCAA
-3') and cloned into pDONR221 by BP recombination. nlsGFP was amplified with primers (forward: 5'-GGGGACAGCTTTCTTGTACAAAGTGGAGATGGAGCAGAAGCTGATCC-3' and

reverse: 5'-GGGGACAACTTTGTATAATAAAGTTGCTACCCGGACTTGTACAGCTC-3') and cloned into pDONR P2R_P3 by BP recombination. *CRE1::SHRΔNLELDV:nlsGFP* was constructed into dpGreen-BarT by Multisite gateway LR recombination. The clone in the binary vector was transformed into *Agrobacterium* GV3101 with pSOUP [51] and transformed into *shr-2*.

3. *PHB*:: *phb-7d:GFP*

To generate *PHB*::*phb-7d:GFP* the *PHB* promoter in *PHB::PHB:GFP* [27] was PCR amplified using the primers 5′-ggtaccCGGAAAATGACACCAACAAG-3′(PHB.KpnI.F) and 5′-GTCGACagctcaaagtcagaaatagg-3′(PHB.SalI.R) and subcloned into the *Kpn*I and *Sal*I restriction sites of pBluescript II (pBSII). The first 647bp of the *phb-7d* coding sequence (including the mutated miRNA recognition site) was PCR amplified with the primers 5′-gtcgacATGATGATGGTCCATTCGATGAGC-3′ (PHB.SalI.F) and 5′-CTGCAGTTGCGCGAAATAGCG-3′ (PHB PstI.R) using cDNA generated from *phb-7d* mutants and subcloned into the *Sal*I and *Pst*I sites of pBSII containing the *PHB* promoter. To obtain the *PHB*:: *phb-7d:GFP* construct, the *PHB* promoter and *PHB* coding sequence until the intragenic *Pst*I site from the *PHB::PHB:GFP* construct was replaced with the *PHB* promoter plus *phb-7d* sequence from the pBSII construct using the *Kpn*I and *Pst*I restriction sites.

*4. CRE1::PHB-m:GFP*

PHB-m harboring a point mutation in the microRNA complementary site (Supplementary Fig. S10) was created by primers (forward 5'-GGATGAAGCCTGGACCGGATTCTATTGGC-3'and reverse 5'-GCCAATAGAATCCGGTCCAGGCTTCATCC-3') with the Quik-Change II site-Directed Mutagenesis Kit (Stratagene) using *PHB* cDNA in pDONR221 as template. *CRE1::PHB-m:GFP* with nos terminator was constructed by Multisite gateway LR recombination into dpGreen-BAR. The clone in the binary vector was transformed into *shr-2 phb-6* and wt.

5. miR165/6 sensor GFP

ER-GFP harboring miR165/6 target sequence of PHB in 3'UTR was generated by amplifying ER-GFP with primers (5'- CACCATGAAGACTAATCTTTTTCTC -3' and 5'-

TAAGGATCCATCCGGACCAGGCTTCATCCCAATAATTTAAAGCTCATCATGT TTG -3') and directionally cloned into pENTR-D-TOPO vector. An intergenic region of *At2G27510* (U2) was cloned into pDONR P4_P1R by BP recombination. This gene was selected as potentially ubiquitously expressed based on the root expression data [45]. U2 promoter was fused to GFP by Multisite gateway LR recombination into dpGreen-BarT. The clone in the binary vector was transformed into Agrobacterium GV3101 with pSOUP [51] and transformed into Columbia wild type. To examine the sensor GFP expression in *shr* mutant, the transgenic plants were crossed to *shr-2* and F2 plants were imaged.

*6. miR165/6 promoter::GFP*

Complete upstream intergenic regions of 8 of the 9 genes encoding *miR165/6* (except *165b*) were cloned into pDONRP4_P1R by BP cloning. Promoters were fused to the ER-localized GFP:NOS terminator and cloned into dpGreen-BAR [27] by LR cloning. The primer information of the promoters is below.

Primer information for the isolation of *miR165/166* promoters

| Primer name | Sequence |
|---|---|
| miR165a-F-attB4 | GGGGACAACTTTGTATAGAAAAGTTGTGTCAGTGCATGGATGTTTT |
| miR165a-F-attB1 | GGGGACTGCTTTTTTGTACAAACTTGGGTGTTATGATGAGGGAAT |
| miR166a-F-attB4 | GGGGACAACTTTGTATAGAAAAGTTGGGAATTTTTCCTCAGAGG |
| miR166a-R-attB1 | GGGGACTGCTTTTTTGTACAAACTTGAAGAAGATAAGTGAGTGGTGAT |
| miR166b-F-attB4 | GGGGACAACTTTGTATAGAAAAGTTGTCTTCCATGGTTTTCACCAG |
| miR166b-R-attB1 | GGGGACTGCTTTTTTGTACAAACTTGGTGATGATGAAGAGAATGAT |
| miR166c-F-attB4 | GGGGACAACTTTGTATAGAAAAGTTGTTTCACTTGTAGAAATCAAAAG |
| miR166c-R-attB1 | GGGGACTGCTTTTTTGTACAAACTTGGAAGAGAAAGAAAGGTATGT |
| miR166d-F-attB4 | GGGGACAACTTTGTATAGAAAAGTTGATTTGGAAGAACAAATTAAG |
| miR166d-R-attB1 | GGGGACTGCTTTTTTGTACAAACTTGGAAGAAGAAGAAGAAGAAGCT |
| miR166e-F-attB4 | GGGGACAACTTTGTATAGAAAAGTTGGAGCTATGAATCTTGCCTTT |
| miR166e-R-attB1 | GGGGACTGCTTTTTTGTACAAACTTGCTCGTGCCAGACAACATTCCCC |
| miR166f-F-attB4 | GGGGACAACTTTGTATAGAAAAGTTGTGCAAACCCTCTTTCTCATC |

| | |
|---|---|
| miR166f-R-attB1 | GGGGACTGCTTTTTTGTACAAACTTGCGAGCCAGGCATCATTCACC |
| miR166g-F-attB4 | GGGGACAACTTTGTATAGAAAAGTTGAGAAGAAACAGAGAGAGAAAG |
| miR166g-R-attB1 | GGGGACTGCTTTTTTGTACAAACTTGCGAGCCAAACAACATTCCTC |

7. *CRE1::MIR165a*

The *CRE1::MIR165a* construct was generated by amplifying a 318bp PCR product containing the *MIR165a* stem sequence from genomic wt DNA using primers 5´-ataGGTACCgtaaatctactcttaagaagc-3´ and 5´-ataGGATCCctacaacaaaaatttgtgaatctgc-3´. *MIR165a* was then cloned into the *BamH*I and *Kpn*I site between a 2.7kb *CRE1* promoter sequence and nosT of the pWLO1 vector [35].

*8. SCR::MIR165a*

The *SCR::MIR165a* construct was generated by cloning *SCR* 5' sequence and *MIR165a* genomic DNA sequence into pBm43GW [52] using standard methods with primers pSCR_LP: GGATAAGGGATAGAGGAAGAGG, pSCR_RP: GGAGATTGAAGGGTTGTTGGTCG, miR165a_LP: CTACAACAAAAATTTGTGAATCTGC, and miR165a_RP: AAATCTACTCTTAAGAAGC via pDONR vectors (Invitrogen). The *SCR::MIR165a* construct was introduced into *scr-6*.

*9. UAS::miR165A*

Genomic sequence of *MIR165a* was amplified using miR165A-F (5'-CACCGCTATTTCAGTTGAGGGGAATGTTGT-3') and miR165A-R (5'-TCAGAGGCAATAACATGTTGGGGG-3'). The amplified DNA fragment was directionally cloned into pENTR-D-TOPO vector. 6xUAS was cloned into pDONRP4_P1R and fused to *MIR165A* by Multisite gateway LR recombination into dpGreen-BarT. The clone in the binary vector was introduced into *J0571; shr-2* and *J0571; scr-4*.

**Genetics**

The *phb-7d* mutant was isolated in a screen for altered expression of the *AtSUC2*::GFP marker line [41]. Seeds of this line (C24 ecotype) were mutagenised by treatment of 0.3% EMS. A population of ca 7200 M1 plants was generated and seeds collected from plants pooled 30 by 30 generating ca 230 pools of M2 seeds. 800 to 2000 M2 seedlings from

each pool were screened for altered GFP-expression under a Leica stereomicroscope. The *phb-7d* mutant was back crossed to the original *AtSUC2*::GFP line and then crossed to L*er* and Col for genetic mapping. Both mapping populations were used in parallel and a mapping position between CAPS-marker TEN5 (14.3 Mb 1 recomb./158 chr.) and the SSLP marker SNP9378 (15.7 Mb; 9 recomb./180 chr.) was established using standard SSLP markers, or ecotype polymorphisms, to create new SSLP markers. The mapping window surrounded *PHB* at 14.6 Mb, which was sequenced using standard methods.

The *scr-6* mutant allele was selected from a genetic screen for down regulation of p*AHP6*::GFP [35] expression, and was identified by standard genetic mapping procedures.

For co-segregation analysis of *shr-2* J0571 rescued by 6xUAS:MIR165A this line was crossed with *shr-2*. Segregation of J0571 GFP, root length and xylem phenotype was analyzed in the F2 generation.

*phb-6 shr-2* was isolated from the segregating quadruple mutants, *phb-6 phv-5 rev-9 shr-2* by PCR genotyping. The primer information is listed below.

Primer information for genotyping *phb-6 phv-5 rev-9 shr-2*

| | primer name | sequence |
|---|---|---|
| genotyping *shr-2* | wt | |
| | 1670F | GACACTGCACGTGCGCAACA |
| | SHR-2RN | TGTCGTCTGATCTTGTGGCTAAAG |
| | shr-2 | |
| | SHR-2FN | CTGAGGTTAGGATTAGACCATTGG |
| | SHR-2RN | TGTCGTCTGATCTTGTGGCTAAAG |
| genotyping *phb-6* | wt | |
| | Phb-6F2 | TCGAGATTGGCGTCTGAGATAAA |
| | Phb-6R2 | TTGGAAACGCATTCAAAGACAAT |
| | phb-6 | |
| | Phb-6F2 | TCGAGATTGGCGTCTGAGATAAA |

| | | |
|---|---|---|
| | Ds 3'-1 | GGTTCCCGTCCGATTTCGACT |
| genotyping *rev-9* | wt | |
| | Rev-9F2 | CCTCTGTTCCAAAGTTCAGCAG |
| | Rev-9R | CGACCCTCAAAAAAAGTCTCAAACT |
| | rev-9 | |
| | Rev-9F2 | CCTCTGTTCCAAAGTTCAGCAG |
| | GUS_R2 | GCCTGCCCAACCTTTCGGTA |
| genotyping *phv-5* | wt | |
| | phv-F | GTTCCTTGTCCTTTCTCTCTCAG |
| | phv-5R | GTTTGATTAGCTGTCACTTTTCC |
| | phv-5 | |
| | phv-F | GTTCCTTGTCCTTTCTCTCTCAG |
| | Ds 5'-1 | ACGGTCGGGAAACTAGCTCTAC |

The quintuple *athb8-11 cna-2 phb-13 phv-11 rev-6* mutant was generated by crossing *athb8-11 cna-2 phb-13 phv-11* and *cna-2 phb-13 phv-11 rev-6/+*. Segregating mutants producing one or two radialized cotyledons and no apical meristem in the F3 generation were genotyped as described [33] and further analyzed. Seeds for loss-of-function *HD-ZIP III* mutants and multiple mutant combinations were a generous gift from S. Clark and M. Prigge.

GFP and GUS cell and tissue markers were introduced into mutant backgrounds by crossing or transformation using the floral dip method [40]. Analyses were done on segregating F2 generations after crosses if parental plants were in different ecotype backgrounds. Histochemical analyses of GUS were as described [53].

**Measurement of root length and meristem size**

Root length of 6 day-old seedlings was measured from 52 individuals of a T3 line of *CRE1::SHRΔNLELDV:nlsGFP* and 39 individuals of *shr-2*. The meristem size was measured by counting the number of ground tissue cells not yet elongated. 18 individuals from a T3 line of *CRE1::SHRΔNLELDV:nlsGFP* and 12 from *shr-2* were

imaged under the confocal microscope for the measurement of meristem size. Student t-test was performed using JMP.

## Supplementary Notes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